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Editorials from The Journal of the National Cancer Institute

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Editorials from

The Journal of the National Cancer Institute

John C. Bailar III, M.D., Ph.D.
Editor in Chief

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FOREWORD

In 1971, the *Journal of the National Cancer Institute* published the first in a series of editorials dealing with various aspects of cancer. The series was conceived by Dr. Mearl Stanton, then Editor in Chief of the Journal, and members of the Editorial Board as a way to highlight some aspects of cancer research and of thinking about cancer research that might not otherwise come to the attention of the general cancer research community. Except for four cases apparently overlooked by the Editorial Office, the first page of each Editorial has included the following note:

> Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

The final words of that statement emphasize the dedication of the Journal to the solution of fundamental problems of cancer as they affect human beings. That dedication is also apparent in the fact that 34 of the 73 editorials deal directly with human cancer, and each of the others has an easily traceable link to the prevention or treatment of cancer in human beings.

Most of these were published as Guest Editorials, the rest as unmodified Editorials. The distinction generally indicated only whether the senior author was a member of the *Journal of the National Cancer Institute* Board of Editors at the time the Editorial was published, but Journal policy on this matter changed from time to time and there were a few exceptions in both directions. Such changes seem to be the editorial analog of Brownian motion.

Nearly all Editorials were prepared at the specific request of the Editor in Chief acting on behalf of and at the request of the Board. The others were voluntary contributions accepted with the express approval of the Board.

These reprinted Editorials are arranged by date of original publication within broad categories. Most were easily classified, despite numerous references to or implications for research fields beyond the main point of discussion. It seemed that this arrangement would be more useful to readers looking for specific items than arrangement by date of publication, name of senior author, or in some other way. However, an author index is included.

The body of this book has been reproduced by photo-offset from back issues of the Journal. Thus numerous changes in Journal style are obvious, though we trust they are not obtrusive. This method of reproduction precludes changes in text. However, the authors of each Editorial were invited to submit a short addendum. These follow each Editorial, unless the authors did not choose to add to their original statement. These addenda have been used variously to update the evidence for or against hypotheses discussed in the text, give credit to other investigators, repair omissions or errors, add references, offer additional perspectives or insights, or comment on the general state of cancer research, medicine, or the universe. I have found them as interesting as the original Editorials.

The primary purpose of this volume is to facilitate reference to a wide spectrum of broad views, or a broad spectrum of wide views, that have been cited in many other publications and, for the most part, continue to be of lively interest. I believe they are of value in other ways, too: as documentation of our rapid progress over a very short time; as a composite record, by leaders in the field, of the reasoning behind many newly developed concepts; and as material for a history of the war against cancer. I believe that other participants in the war will also find that these careful expositions of personal views continue to be as fascinating, stimulating, and humbling as when they were first published. There is still a long way to go.

John C. Bailar III
Editor in Chief
Journal of the National Cancer Institute

PART I: HUMAN STUDIES

General Epidemiology of Human Cancer

Human Carcinogenesis: Chemical and Physical Agents

Human Carcinogenesis: Drugs and Hormones

Human Carcinogenesis: Microbiologic Agents

Lung Cancer in Man

Large Bowel Cancer in Man

Treatment of Human Cancer

Other Aspects of Human Cancer

GUEST EDITORIAL

by **Denis P. Burkitt**
Medical Research Council
London W1, England

JNCI 47:913–919, 1971

Some Neglected Leads to Cancer Causation

IN ANY SPHERE of life, things dependent on common or related factors tend to occur together and have their highest frequency in regions where the factor or factors on which they depend are present to a high degree.

Birds, plants, and insects that particularly rely on fresh water are found together and in profusion near lakes. Similarly, fauna and flora, which thrive on a combination of rain and heat, exist together in the moist tropics. Biological organisms of all kinds preferring salt water likewise keep company near the seashore.

Human diseases follow the same pattern, tending to cluster within the environment on which they depend—that containing their causative agents. Recognition of the company kept by one disease, *i.e.*, the other conditions associated with it, can be a guide to the factor or factors responsible for its causation.

Before exploring paths suggested by associations between various diseases, both benign and malignant, I will enumerate some basic assumptions on which deductions with regard to relationships will be made.

Basic Assumptions

When two or more diseases have a common or related cause, not necessarily sole cause, they tend to be associated *a*) in geographical distribution. Both or all will be rare or common in the regions where their causal environment is rare or common; *b*) in individuals. The diseases will tend to occur together in patients who have been maximally exposed to the common or related etiological factors.

Conversely, when two or more diseases are recognized as associated in geographical distribution or in individuals, they may be suspected of having common or related causes.

A relationship between two or more diseases, each or all of which can be shown to be connected with a common factor, does not necessarily imply that this factor is causative, but the observation of such an association may provide a hypothesis that can be tested for causal relationship.

When two conditions have been observed to be associated with one another, particularly when one precedes the other in time, the relationship is often wrongly interpreted as one of cause and effect, whereas the correct deduction should perhaps more often be that of a common or related cause (text-fig. 1).

Examples of Association of Diseases With a Common or Related Cause

The relationship between lung cancer and bronchitis is an obvious example of two diseases associated both in geographical distribution and in individuals because they are due to a common

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Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

cause—tobacco smoke (*1*). Similarly, skin epitheliomas, rodent ulcers, and loss of skin elasticity are related because the development of both these tumors and the loss of subcutaneous elastic tissue result from the same cause—excess exposure to sunlight.

It is less easy to cite examples of diseases that are associated because they result from causes related to each other. One example is the association between lung cancer and edentulous mouths, which might be expected in migrant laborers who have returned to neighboring countries from the South African mines. In the mining communities they have had much greater opportunities for smoking cigarettes and also for consuming sugar than they have in their home locations. These two commodities known to cause bronchial carcinoma and dental caries, respectively, are both more readily available in the mine situation than in the workers' home villages.

Examples of Recognized Association Assisting the Discovery of a Cause

Recognition of related effects has in many instances led to the subsequent detection of the common causative factor. The association of the multiple diverse manifestations of syphilis, including ulcers, skin rash, palate perforation, and sabre tibias, enabled the recognition that these lesions were due to a common cause before the responsible spirochete was identified.

To cite an example in the field of cancer, the recognition of a relationship between the diverse clinical presentations of what is now known as Burkitt's lymphoma suggested they were not, as previously considered, different tumors, but merely different manifestations of a single disease. Subsequently the similar geographical distribution of tumors in the orbit, jaws, ovaries, kidneys, thyroid, salivary glands, and other sites in children confirmed the conviction that clinical observation had impelled—these were expressions of a common cause.

Complication or Common Cause?

It is universally recognized that nicotine-stained fingers and lung cancer are associated, the former preceding the latter in time. Cigarette smoking is accepted as the common cause, and no one would suggest that the cancer was a complication of the stained fingers (text-fig. 1a).

Similarly, in poliomyelitis, paralysis may follow the febrile episode, but the former is considered, not as a complication of the latter but as another effect of the same virus infection (text-fig. 1b).

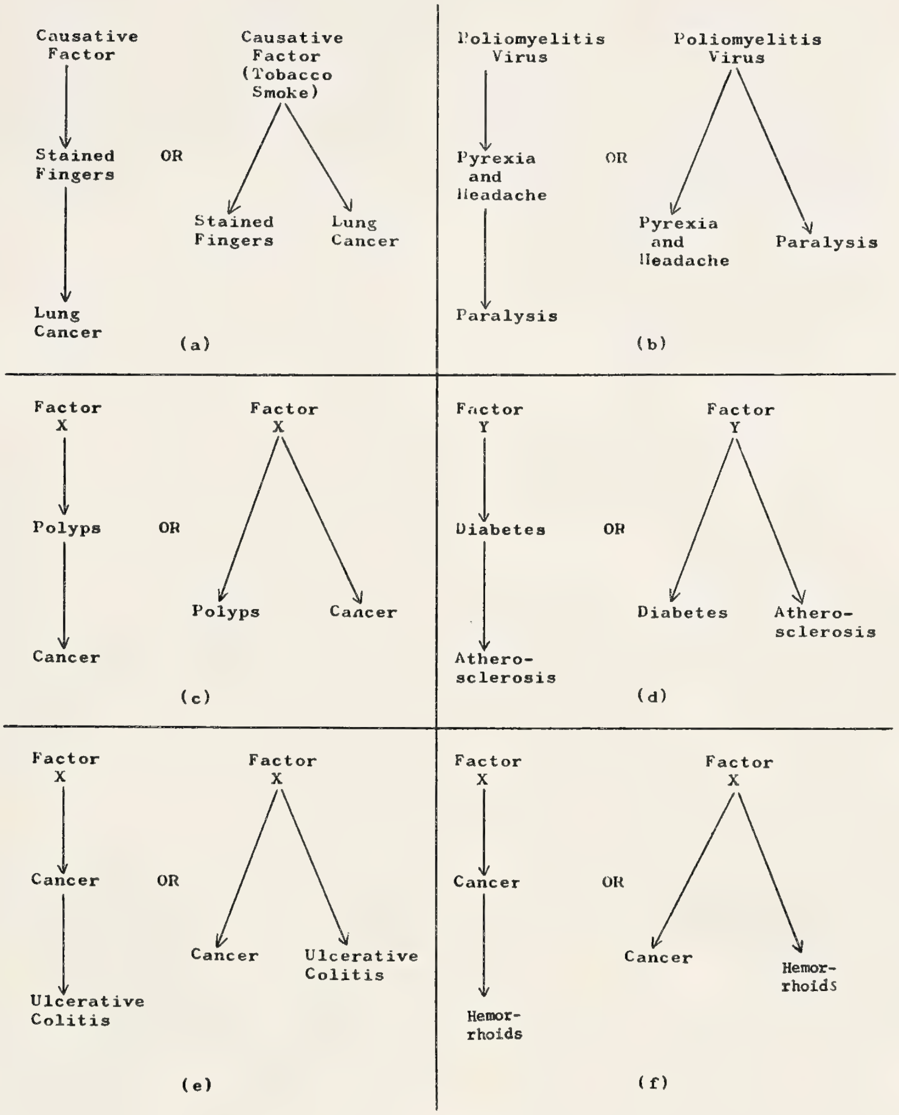
It is becoming increasingly accepted that both polyps and cancer of the large bowel are merely different manifestations of a common cause, and the fact that individual polyps may become malignant may be largely of academic interest. Not only are malignant and benign bowel tumors connected with one another both geographically and in individuals, but, in addition, both have a similar anatomical age and sex distribution and are produced experimentally by the same means (text-fig. 1c).

Many conditions are generally believed to be complications of diseases with which they are associated, whereas the more correct interpretation might well be that they are related merely because they are but different effects of the same cause.

The relationship between diabetes and coronary disease is a good example (text-fig. 1d). These conditions are associated both geographically and in individuals, and Cleave (*2*) and Cleave and coworkers (*3*) might well be right in considering both to be results of overconsumption of refined carbohydrate, though this does not rule out the additional influence of secondary factors. The differing time factor involved in the appearance of each could account for the fact that, in individual patients, diabetes precedes arterial change, and hence the latter is considered a complication of the former. This time factor is also evident in the geographical distribution, since the incidence of diabetes in a community always rises before that of atherosclerosis.

The same argument holds for the association between obesity and diabetes. Does obesity predispose to diabetes or are they both results of the same cause? The epidemiological evidence fits Cleave's suggestion that the latter is true.

To return to cancer, it is generally assumed that the high risk of colon cancer in patients with ulcerative colitis indicates that the former is a complica-


Causative Factor
Stained Fingers
Lung Cancer
OR
Causative Factor (Tobacco Smoke)
Stained Fingers
Lung Cancer
(a)
Poliomyelitis Virus
Pyrexia and Headache
Paralysis
OR
Poliomyelitis Virus
Pyrexia and Headache
Paralysis
(b)
Factor X
Polyps
Cancer
OR
Factor X
Polyps
Cancer
(c)
Factor Y
Diabetes
Athero-sclerosis
OR
Factor Y
Diabetes
Athero-sclerosis
(d)
Factor X
Cancer
Ulcerative Colitis
OR
Factor X
Cancer
Ulcerative Colitis
(e)
Factor X
Cancer
Hemor-rhoids
OR
Factor X
Cancer
Hemor-rhoids
(f)

Text-figure 1.—Common or related causative factors in indicated diseases.

tion of the latter (text-fig. 1e). But no other chronic ulcerative condition of the bowel, such as amebiasis or schistosomiasis, remotely affects the cancer incidence. Is there, in fact, any ulcerative condition that has a marked tendency to undergo malignant change? Epitheliomas developing at the site of chronic leg ulcers in the tropics are probably due to the effect of sunlight on the depigmented scars, rather than examples of malignant change in an ulcer. The observation that ulcerative colitis is exceedingly rare in all communities with a low incidence of bowel cancer and vice versa suggests some related common factor, which may of course be only one of many. The relationship between bowel cancer and hemorrhoids can be similarly explained (text-fig. 1f), and this is consistent with the tentative hypothesis for the cause of bowel cancer outlined below.

Etiological Leads From Well-Known to Less-Known Diseases

It is evident that the recognition of an association between two or more conditions either in geographical distribution or in individual patients, or in both, may provide important guides to some of the factors responsible.

If a condition whose cause is known is observed to be connected with a disease of unknown etiology, the former could be used as a guide to the cause of the latter. The close relationship between diverticular disease and bowel cancer is a good example. The former is almost unknown in communities where bowel cancer is rare, but it is the commonest disease of the bowel in economically developed countries with the highest incidence of bowel cancer. The relationship between diverticular disease and deficient fiber in the diet can be considered established (*4*), and the possible connecting links between this and bowel cancer will be discussed later.

Any search for the cause of cancer of the gallbladder will inevitably be a search for the cause of gallstones, since this form of cancer is almost unknown in the absence of cholelithiasis. There is, moreover, some evidence that the prevalence of gallbladder cancer may be particularly high in regions where gallstones are frequent, but where facilities for their removal are poor. Strong (*5*) sees approximately 15 cases a year in a 150-bed mission hospital in northeast India, a phenomenally high figure compared to the almost total absence of this disease in sub-Saharal Africa.

Investigating the Common Rather Than the Rare Cause

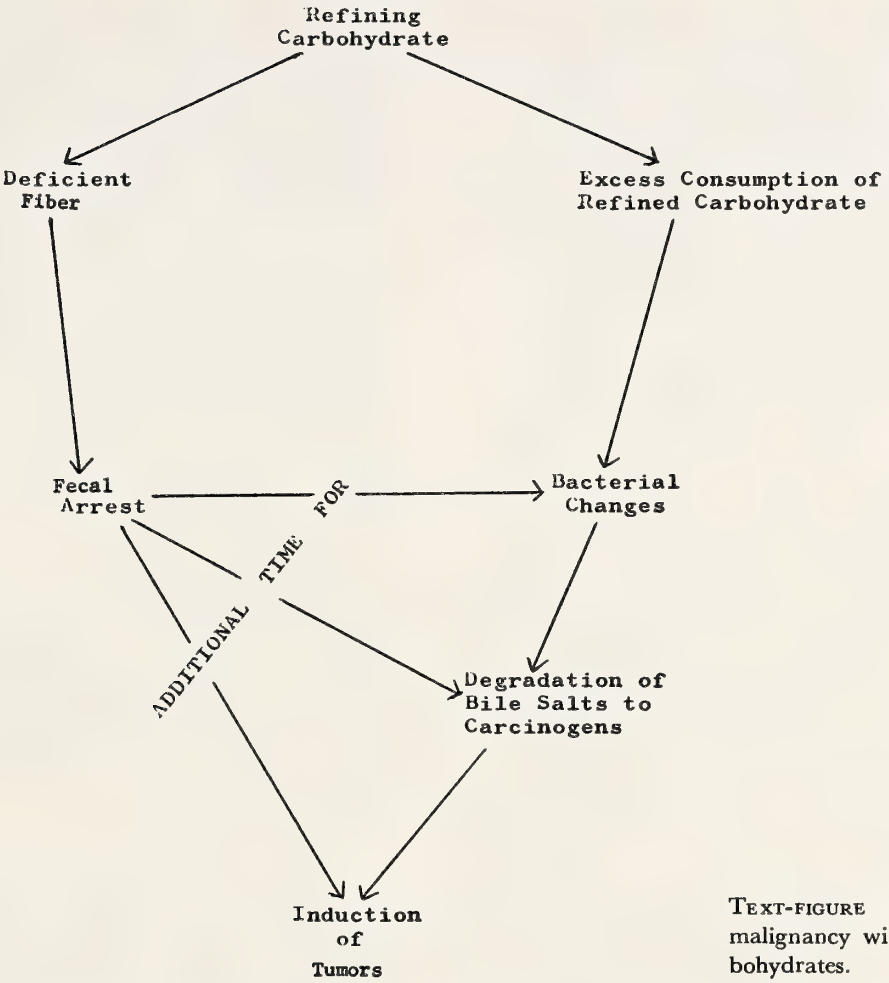
If two conditions of obscure cause are observed to be associated, it would seem reasonable to search first for the cause of the one commoner or more easily detected. The easiest way to find a carcass in the African bush is to look for the telltale vultures flying overhead. This may have an application with regard to benign and malignant tumors, both of which, in particular organs, may well have common causative factors. This is suggested by the observation that, whereas breast cancer is much rarer in African women than in American Negroes, benign breast tumors appear to be relatively even less common in Africa. The same applies to tumors of the large bowel, cancer being rare in Africa and adenomatous polyps almost unknown (*6*).

Cleave's Hypothesis and Bowel Cancer

When Cleave (*2*) formulated his ingenious hypothesis linking many diseases characteristic of economic development to the refining of carbohydrate foods, he unwittingly threw light on the possible etiology of the malignancy responsible for more deaths than any other in the western world except for bronchial carcinoma. He recognized that some conditions characteristic of more affluent communities, including atherosclerosis, obesity, dental caries, diabetes, diverticular disease, appendicitis, and varicose veins, only became common after western patterns of diet were adopted. Since the major dietary changes in the more economically developed countries, during the period in which these diseases have become common, concern the carbohydrate rather than the fat or protein fractions of food, he sought an explanation for the diseases associated geographically and chronologically with changes made in carbohydrate foods. The massive refining of carbohydrates, a change introduced in Europe and North America mainly during the final quarter of the last century, seemed to him a likely factor (text-fig. 2). He considered the refining of sugar, with almost total removal of fiber and protein from the natural product, the chief cause, but also took into account the replacement of whole meal by refined flour. The diminished consumption of other high fiber content foods, such as oatmeal and vegetables, must also be considered.

The most brilliant aspect of his hypothesis is the recognition that different diseases associated geographically and chronologically to carbohydrate refining relate to different effects of the refining process. Thus he attributes diabetes, obesity, and atherosclerosis to the inevitable overconsumption of the refined product, when bulk is reduced by removal of fiber (text-fig. 2). Diverticular disease and other effects of constipation are explained on the basis of fiber depletion.

Carbohydrate foods were incriminated rather than fats or proteins for the following reasons: 1) During the time the diseases in question rose steeply in frequency in North America and Europe, the major food change was the refining of carbohydrates. 2) The geographical distribution of these diseases fits more closely with areas of refined carbo-



TEXT-FIGURE 2.—Association of malignancy with refining of carbohydrates.

hydrate consumption than of high fat or protein intake. 3) Consumption of refined carbohydrate is the only dietary pattern that will explain the relationships between all the diseases listed.

These "western" diseases are certainly associated geographically and in many instances tend to be related to one another in individual patients; my epidemiological studies in Africa and elsewhere substantiate Cleave's basic hypothesis.

Changes made in carbohydrate food may of course be only one of many etiological factors, but in some instances they would appear to be the major one.

Leads to the Cause of Bowel Cancer

To Cleave's list of diseases associated with western diet, only some of which I have included in this discussion, I would like to add cancer of the colon and rectum, and to suggest mechanisms that could explain why bowel cancer should be related to these other diseases.

The association between cancer and other noninfective diseases of the bowel and certain other diseases believed to be diet-dependent obviously makes food the prime suspect when attempts are made to identify a common or related cause. Moreover, the diet most determines the content of the feces which is the most important environment of bowel mucosa. The significance of intestinal content in the causation of bowel cancer is suggested by the observation that in experimental animals loops of bowels, from which the fecal stream has been diverted, remain free of disease when tumors are induced in the intestine (*7*). Further evidence incriminating food is found in the fact that the wide gap between the incidence of bowel cancer in U.S. Caucasians and Negroes, which existed half a century ago, and the similar gaps between the incidence of other conditions, such as diverticular disease, appendicitis, peptic ulcer, and coronary disease postulated to be diet-related, were bridged simultaneously when the differences in the dietary habits of the two races largely disappeared.

Similarly, Japanese emigrants to North America have shown a marked increase in incidence of bowel cancer and other diseases of affluence after their change from traditional Japanese food to the American pattern (*8*, *9*).

That these diseases are rare in communities with a high-residue diet, whereas they are common in communities that have changed to a low-residue diet, suggests that fiber content of diet may be a significant etiological factor (*10–12*).

It has been repeatedly demonstrated that intestinal transit time and stool bulk and consistency are closely related to the amount of unabsorbable cellulose in the food (*11–14*). Low-residue diets are responsible for the fecal arrest and resultant small, firm stools that lead to high intracolonic pressures and thus cause diverticula (*15*, *16*) and possibly also appendicitis (*17*, *18*).

A refined, fiber-depleted diet not only changes bowel physiology but is also related to the bacterial flora of the feces that differs between communities with high and low bowel cancer incidence (*19*, *20*). The altered bacteria are believed to produce enzymes that cause degradation of bile salts with formation of known carcinogens detectable in the stools (*20*, *21*). Moreover, Antonis and Bersohn (*22*) have shown in South Africa that a greater quantity of bile salts can be recovered from the stools of Bantu subjects on a high-residue diet than from Caucasians on a low-residue diet.

Whether the bacterial changes are mainly related to alteration in the carbohydrate, fat, or other content of the food, or to the intestinal arrest caused by fiber depletion has not yet been determined. Carcinogens found in the stools of individuals on a low-residue diet would be held in contact with the bowel mucosa for a longer time and in a more concentrated form than in those consuming a high-residue diet, and the carcinogenic potential might well be a multiple of these two factors (*23*).

The hypothesis outlined above makes it possible to understand why bowel tumors should be associated, not only with diverticular disease but also with hemorrhoids, since the latter are possibly due to the straining at stool inevitably related to constipation. The more distant relationships of diseases unconnected with the bowel can be readily explained by Cleave's hypothesis which connects diseases caused by carbohydrate excess with those caused by fiber depletion.

It is not suggested that the diseases characteristic of economic development are due solely to the cause postulated, though in many instances refining of carbohydrate seems likely to be the main cause, with other factors contributing.

The conditions covered by Cleave in his hypothesis are all considered by him to be manifestations of a single "saccharine disease," meaning related to sugar.

As Cleave has emphasized, in different people certain organs or tissues will be more or less susceptible to different environments, and this accounts for the varying clinical manifestations that may be caused by the same environmental factors in different individuals.

"Blinkered" Research

From these observations one wonders whether cancer research is sometimes too "blinkered" in that it often tends to be focused only on malignant lesions to the exclusion of other conditions, the investigation of which might point the way to a better understanding of certain forms of cancer.

While there has been an enormous and expensive development in tools and techniques to explore intracellular constructions and mechanisms, there has been perhaps neglect of the wider view that sees cancer within the context of other associated diseases.

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ADDENDUM

For most of what I wrote over 7 years ago I would still stand by today. However, I would add to my emphasis on the importance of relationships, not only between a disease and environmental factors but also between one disease and another, the significance of a lack of relationships.

Whereas an association between a disease and environmental factors that have been postulated to be causative of the disease does not *prove* a cause-and-effect relationship, the absence of any consistent relationship between a disease and a supposed cause goes a long way to *discount* a hypothesis. A major role of epidemiology is to eliminate untenable hypotheses and thus reduce the number of those deserving experimental verification.

The direct relationship between polyps and cancer of the colon is generally accepted and many authorities (*1*) go so far as to suggest that in western countries all cancers arise in polyps, the sequence being small polyps to large polyps to cancer, possibly with different environmental factors responsible for each stage of the process. This sequence can only account for the colon cancers in western countries that are in addition to the small number observed in rural communities in developing countries, since polyps are virtually unknown in these situations.

I would now doubt whether cancer and ulcerative colitis share a common cause despite their close epidemiologic association. I have come to view fiber-rich plant foods as *protective against* a number of related diseases, all of which may have different causative factors rather than considering the *lack* of fiber as causative of these diseases. Since fiber is known to neutralize certain toxins in the bowel (*2*), it could presumably be protective against ulcerative colitis whatever its cause. Some clinics are successfully using high-fiber diets in the treatment of this disease.

In the past, I minimized the marked changes in fat intake that distinguish between diets of those people living in third world countries and in more affluent societies. These contrasts are similar to those between western countries a century ago and today. Since the intakes of fat and fiber are almost invariably inversely related to one another, both must be considered. Fat may be causative of, whereas fiber, particularly that from cereals, may be protective against coronary heart disease (*3*) and colon cancer (*4*).

Results of studies on fiber have been significant since 1971. No longer can we refer to it as a single substance, and the term crude fiber is seen to be grossly misleading. Different components of what is now referred to as dietary fiber are probably protective against each of the so-called western diseases that have been observed to be associated with one another.

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EDITORIAL

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Transplacental Chemical Carcinogenesis in Man

A MOST dramatic development in cancer research was the report last April that synthetic stilbestrol therapy during pregnancy induced vaginal cancer in the child 14–22 years later (*1*). This observation represented the first evidence of transplacental carcinogenesis in man.

Herbst *et al.* (*1*) reported 8 young women with clear-cell adenocarcinoma of the vagina, a neoplasm extremely rare so early in life. All but 1 were seen at a single hospital. Seven of their mothers had received stilbestrol therapy for threatened abortion during pregnancy. None of the 32 matched control mothers had been so treated. The relationship was quickly confirmed by data from the New York State Tumor Registry (*2*), which revealed 5 cases, all following heavy therapy with synthetic estrogens during pregnancy. The doses ranged up to 125 mg/day. Herbst *et al.* (*3*) stated that they have learned of more than 20 cases recognized since their first 7 cases were described.

An experimental study 8 years before suggested that immaturity may heighten susceptibility to cancer induction by stilbestrol. Dunn and Green (*4*) observed cancer of the vagina and other sites after a single dose of diethylstilbestrol to mice at birth. The human counterpart, however, was recognized only because an extremely rare form of neoplasia was observed in a time-space cluster among adolescent girls.

Other evidence of prenatal determinants of cancer in man include *a*) the occurrence of congenital cancers—cancers that grow to large size *in utero* (*5*, *6*); *b*) peaks in mortality soon after birth, primarily for cancers of the same cell type as occur congenitally (*e.g.*, teratoma, neuroblastoma, primary liver cancer, Wilms' tumor, and leukemia) (*7*); *c*) the excessive concurrence of certain cancers and congenital malformations (*7*), the latter having originated, of course, during embryogenesis; and *d*) an increase in childhood leukemia related to high maternal age or low birth order (*8*). Whether chemicals traverse the placenta to produce these effects is unknown.

Prenatal determinants in which the mode of transmission is known may involve *a*) germ cells—genetic as in retinoblastoma (*9*) or von Recklinghausen's disease (*10*) or cytogenetic as in the association between leukemia and Down's syndrome (*11*); *b*) transplantation—metastases from mother to child (*12*) or from one identical twin to the other while sharing the placental circulation (*13*); and *c*) ionizing radiation, an external agent that passes through the mother's abdominal wall to reach the fetus (*14*, *15*).

Thus cancer may in various ways be inborn. The first reports of transplacental chemical carcinogenesis raise 3 questions: *a*) What other cancers are induced by the same agent (stilbestrol); *b*) what other clinically used chemicals cross the placenta and may be oncogenic, and *c*) how can cancers so induced be recognized?

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The study of mice by Dunn and Green (*4*) indicated that stilbestrol can induce not only vaginal cancer but also cancer of the uterine cervix and granular cell myoblastoma. Male mice revealed an excess only of multiple cysts of the epididymis. These and other disorders may be sought by follow-up study of hundreds of children born of mothers treated with large doses of stilbestrol during pregnancy. The results will not be known for several years. Meanwhile, it would be valuable to seek quick (though incomplete) answers by linking obstetric hospital records with tumor-registry data when feasible. The opportunities to do so are limited to those few areas of the United States with tumor registries. The same approach for diseases other than cancer is even more restricted. The need to increase the capacity for medical record linkage in this country is obvious (*16*).

What other chemicals may sow the seeds of cancer during fetal life? Cytotoxic anticancer drugs are suspect. Aminopterin and methotrexate are known to be human teratogens (*17*). Methotrexate is used in the treatment of psoriasis. Other drugs known or suspected to cause cancer in man may, of course, pose a similar threat after fetal exposures. Among the most prominent are immunosuppressive therapy (*18*), Dilantin (*19*), and chloramphenicol (*20*). To this list one may add environmental chemicals other than drugs, some of which are known to harm the embryo by the malformations they produce [*e.g.*, alkylmercury (*21*) or heat transfer agents (*22*)].

In theory, surveillance of cancer occurrence may reveal exceptional clusters. It was not routine surveillance, however, that revealed the oncogenicity of stilbestrol; as usual, clinicians traced a cluster to its cause (*1*). The stilbestrol story will undoubtedly renew interest in monitoring tumor-registry data in the hope of detecting other clusters, which, when studied retrospectively, may lead to agents not now suspect in human oncogenesis. Meanwhile, drugs already under suspicion can be evaluated for transplacental carcinogenicity through prospective (follow-up) studies of children born of women who were exposed to these agents during pregnancy.

Characteristics peculiar to certain stages of fetal development or the environment may increase or decrease the transport of a compound across the placenta. As such information is developed through laboratory research, hypotheses may be generated that can be tested epidemiologically for their application to carcinogenesis in man. The converse may also apply: Observations in man may suggest hypotheses that can be tested in the laboratory. The value of an interaction between experimentalists and epidemiolgists is once again apparent.

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ADDENDUM

In the 7 years since the Editorial appeared, the risk of clear cell adenocarcinoma in young women exposed in utero to diethylstilbestrol (DES) has been estimated from a registry of such cases to be 0.14–1.4 per 1,000 (*1*). The tumors were rare in young women under 14 years of age, then the frequency rose rapidly to a peak at 19 years, followed by a precipitous drop. Maximum use of the drug seems to have been in 1951–53. Transplacental carcinogenesis in experimental animals is observed when exposures occur late in pregnancy, after organogenesis, but the DES-induced cancers in young women occurred only when exposure was early in pregnancy, i.e., during organogenesis. Thus the neoplasia is apparently linked to the much more frequent DES-induced congenital anomalies (adenosis and ridging) in the lower genital tract (*2*). Abnormalities of the genital tract of males exposed in utero to DES have also occurred, but no cancers have been observed (*3*).

No other transplacental human carcinogens have been identified, but one is strongly suspected. Diphenylhydantoin (DPH), taken for epilepsy, has been associated with lymphoma. When used during pregnancy, it can produce malformations in the child, such as typically mid-facial hypoplasia and aplasia of the fingernails and toenails. Therefore, it was thought that DPH taken during pregnancy might transplacentally induce lymphoma in the offspring, and it came as a surprise when 2 children with the fetal hydantoin syndrome were described not with lymphoma but with neuroblastoma [reviewed by Miller (*4*)].

The possibility that immunosuppressants given during pregnancy might induce lymphoma in the offspring has fortunately not been observed, even when given in conjunction with renal transplantation (*5*, *6*), a powerful lymphomagenic circumstance (*7*). Among other possible maternal exposures during pregnancy that may predispose the child to neoplasia are the use of alkylating agents for the treatment of disease (*8*) or occupational exposure to benzene (*9*). In this connection, it is interesting that gas chromatographic–mass spectrometric analysis of low-molecular weight, volatile organic substances in umbilical cord blood and maternal blood samples revealed that more than 100 components were present in the cord blood in significantly higher concentrations than in the maternal blood. Notable among the chemicals in this study were benzene, carbon tetrachloride, and chloroform (*10*).

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GUEST EDITORIAL

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Epidemiology: Problems in the Study of Cancers of Low Incidence and the Need for Collaboration

MANY CANCERS exist for which there are currently no clear hypotheses that could be pursued in epidemiologic investigations. These include carcinomas of the kidney, some parts of the digestive system, non-Hodgkin's lymphomas, tumors of the nervous system, and many sarcomas, whereas the same lack of etiologic factors is also evident in many common cancers other than those clearly related to smoking or occupational carcinogens. Even so, the incidence of most of them shows substantial international variation (*1*). Although part of this variation may be due to international differences in diagnostic or reporting practices, it is nevertheless important, if we adhere to the environmental hypothesis as an explanation for most of the discrepancies (*2*), that we attempt to identify the relevant environmental factors.

The question arises as to how an epidemiologist obtains appropriate hypotheses and how he secures support for an exploratory study which may be criticized because he does not have a clear hypothesis to test.

Classically, the epidemiologist will proceed by literature review, consultation with clinical and basic science colleagues, and by a search for leads from clinical practice or laboratory investigations, especially if there are appropriate animal models. Descriptive national and international epidemiologic data will be explored over recent and longer time periods. To seek other clues, these data may be analyzed further for cohort differences. Thus the importance of good reliable data is emphasized. Thinking and posing the right question are essential (*3*). But in the absence of an appropriate hypothesis, epidemiologists are driven to perform exploratory studies in search of information on any factor that may be applicable to the condition under investigation. Usually this will involve the case-control or retrospective approach, but occasionally access to existing cohorts with identified information collected before the onset of the disease might justify either a case-control approach within these data or a follow-up cohort study.

Unfortunately, in a case-control study a long time will be required for a study of a rare condition. Furthermore, the nature of the data sought and ideas may change with time so that the investigator may be tempted to include additional requirements for information within the instrument used. If this process continues extensively, the end result may be a data set consisting of information obtained on varying numbers of individuals that may be almost impossible to interpret.

Further, problems may arise when one considers sources of cases and controls. How important is it that they should be representative of the population from which they are drawn? In the past many epidemiologists apparently regarded exploratory studies as justified, albeit they were based on highly selected populations sometimes picked up through a referral center. It may be questioned whether one can justify a study of prevalent cases or new ones coming to a referral center, since the results apply only to patients surviving long enough to be available for study or attending the center for therapy. Thus the extent to which they are representative of all cases cannot be determined. It is important, even in a rare condition, that unselective series are studied, preferably all those that occur in a defined area and time period. Otherwise, whatever the leads, further studies will be needed to confirm the general applicability of the results. An additional advantage of such an approach is that attributable risk estimates (*4*) can be calculated. That is the extent to which a factor, identified as contributing significantly to the occurrence of the disease, is sufficiently important in terms of the numbers of cases induced by it to justify further investigation and preventive measures (*5*).

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What about the controls? Is it ever justifiable to use patients with malignant disease as controls for patients with other malignant conditions? Presumably not, if most cancers are environmentally induced, because these individuals cannot represent the population from which cases are drawn. What about available numbers? Although a number of epidemiologists have increased the control-to-case ratio to compensate in rare conditions for deficiencies of cases, it is not possible if invalid controls are used to compensate for deficiencies in design by this process. Surely, in an investigation of a rare condition, appropriate care must be taken to select controls representative of the population from which the cases are drawn. This is not to imply that controls always have to represent the normal population, even though this should usually be the rule in exploratory studies. Close matching may be required if one is seeking to refine the conclusions from previous studies to avoid confounding (*6*), but unfortunately the opportunities for such precision are rare.

Thus we must make certain we do not overlook elementary precautions of design in the need to ensure economies in exploratory studies which in the past resulted in reports raising more questions than they answer.

What about the analysis? Why do authors so often ignore the matching they have painstakingly done initially, even though the precision of analysis is increased by the retention of the original matching (*7*), whereas to ignore it could be incorrect (*8*)? Surely we should not refrain from attempting to unravel multifactorial causes, particularly since models for multivariate analysis are applicable to case-control studies (*9*).

What about the report? Is it justifiable at this time of information overload for editors to devote space to reports of studies criticized by reviewers for what now seem to be elementary errors of design but which may be based on plans finalized many years before? Editors should neither condone elementary defects in analysis nor permit authors to fail to consider the implications of admitted deficiencies of design. However, albeit some conclusions of authors may be questioned because deficiencies may give rise to many difficulties in interpretation, they still may give other workers the clue to the appropriate investigation. Thus publication is justified at least in a brief form. After all, a study does not live in science until it has been published and can be criticized; editors and reviewers should be careful that their own biases and dogma do not prevent from seeing the light of day what, to their surprise, could be important leads. But authors should be careful not to submit for publication studies, however inconclusive, without at least pointing out a tentative working hypothesis that can be evaluated by themselves or others. A recent example is a study of prostate carcinoma (*10*). Although the work was exploratory and largely negative, it did suggest to the authors a more appropriate age (late middle age) for investigation of possible etiologic factors than they had themselves pursued. As Lowe (*3*) reminded us, a good scientific hypothesis is one that can be disproved, and thus can lead to the substitution of a more satisfactory hypothesis, and so on, until "truth" is finally obtained.

If so many questions can be directed to studies that have been or may be published, does the epidemiologist have a place in attempting to develop hypotheses that he may evaluate in man, or his laboratory colleagues may evaluate in animals before further investigations are contemplated in man? Surely, the answer must be yes. But the epidemiologist in designing studies and the granting agency in funding him do have an obligation to the scientific community to ensure that all available knowledge is taken into account. The International Agency for Research on Cancer is attempting to make certain that appropriate consideration is given to the multidisciplinary approach in investigations, as shown by a conference report which considered environmental causes of cancer (*11*). There is also an increasing interest in multidisciplinary workshops to consider more focused attacks on problems.

Other solutions involve a degree of cooperation and collaboration, possibly foreign to many investigators, and a subjugation of personal aims that scientists, who think of themselves as independent investigators, may find unacceptable. However, lessons have been learned by clinicians in relation to the need to conduct multicenter-controlled collaborative clinical trials; it is time that epidemiologists realized a similar need. With the use of the resources of several centers and with a carefully thought-out common design and instrument, studies of a rare condition will result in more rapid answers than are possible from one institution. This will avoid the tendency to change design or the nature of the data sources as time passes. Such studies need not be onerous for the investigator, because his contribution will be relatively slight, but he will have the intellectual satisfaction of participating in a joint project and achieving answers that he could not get on his own. This approach will be more expensive for the granting agency, but will be worthwhile because it will be more "cost-effective" than alternative approaches. A recent example of such collaboration has been the investigation of the epidemiology of Hodgkin's disease by workers in New Orleans and Los Angeles (*12*) and is also exemplified by current collaborative studies of breast and bladder cancer in Canada.

A further approach is to make sure that unselective cases are studied with the use of data now being accumulated in cancer registries in many parts of the world. The opportunities, now arising in the Program of Cancer Surveillance, Epidemiology, and End Results Reporting of the U.S. National Cancer Institute, should be used for studies within individual component parts of this program and also by collaboration between centers to guarantee that rare conditions are appropriately studied.

There is nothing wrong with our methodology, either case-control or cohort. It is our inability to use the data, ideas, and leads already available to us to investigate the etiology of the conditions we are interested in, using modern methods of analysis to dissociate the contribution of the various factors which together cause the cancer. Indeed, it is possible for many conditions that the clues are already available. To enable us to solve the problems, we need appropriate detective work and alert minds for the unexpected. An example of an unexpected outcome from an exploratory study has just come from the Boston Collaborative Drug Surveillance Program (*13*). Unfortunately, two studies designed to confirm a possible association of reserpine and breast cancer (*14*, *15*) suffered from some of the deficiencies I have discussed so that the reality of the association and possibly more important its significance remain in doubt.

An "ex cathedra" statement sometimes made is that the differences between those that have cancer and those that do not may not be sufficiently great in the Western World for detection of causative factors with the use of standard epidemiologic techniques. This is a belief to which I do not subscribe, though I accept that perhaps in many circumstances our methodology has not been good enough, until now, to enable us to detect the differences that must exist. We have to remember it may be as important to find out why 9 of 10 heavy smokers do not get lung cancer as to identify the 1 of 10 who does. These are the sorts of clues we should follow with as much energy as our searches for factors in individuals who actually develop the disease.

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ADDENDUM

It is paradoxical that one of the studies to which I referred when writing this Editorial 4 years ago should have raised, 3 years later, so many of the issues I had mentioned. Our collaborative study of bladder cancer indicated, with a suggestion of a dose–response relationship, that consumption of artificial sweeteners may be a risk factor for bladder cancer in males (*1*). However, the discrepant findings in females, our use of neighborhood controls, and the weak association all combined to evoke criticism (*2*) and the need for further investigation. This experience confirmed 1) the desirability of having a representative case series, which we largely did; 2) the desirability of having a representative and, in most respects, a comparable control series which most of our critics believed we did not; and 3) the need for collaboration in ensuring that sufficient numbers of cases could be studied, i.e., to be certain that the lack of an effect in females was not an artifact.

A favorable effect of this controversy has been that one of my suggested approaches to collaboration, the utilization of the Program for Cancer Surveillance, Epidemiology and End Results Reporting (SEER) for case identification, has been invoked to initiate in many SEER centers a collaborative study of the epidemiology of bladder cancer. I fervently hope that those conducting a number of studies will use this mechanism.

The controversy has underlined the major difficulty that arises in interpreting assays if there are doubts over the control series. Indeed, I may have been oversimplistic in suggesting in 1974 that *nothing* was wrong with our methodology. However, we remain unrepentant over our attempt to achieve population controls (*3*). Indeed, the alternative espoused by many, i.e., the use of hospital controls, not only might have involved us in further difficulties but may be the reason that other case–control studies (*4*–*7*) of this association were negative. The demonstration that Berkson's bias can indeed operate adds weight to this view (*8*). Nevertheless, it is difficult to ensure that population controls pay as much attention as do patients to the questions asked of them, and we need to develop further methodology to avoid response bias and to correct for it if we have any indication that it may have occurred. What is more, even with the present anxiety over confidentiality, we need to be even more positive in our approach to those who have under their control the sampling frames that would enable *identified* population sampling to be conducted.

In conclusion, it is to be hoped sincerely that the next 4 years will at the least see a resolution of the artificial sweetener controversy, though I fear we may find ourselves with another equally as vociferous. However, the need for painstaking attention to the details of our discipline remains, and it would seem that nearly all I wrote in 1974 still applies in 1978.

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GUEST EDITORIAL **by Sidney J. Cutler**[1]

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Cancer Registries: Opportunities and Responsibilities

For many years, cancer registries have been recognized instruments for the promotion of continuous care of patients, the evaluation of diagnostic and treatment practices, the assessment of end results, and the description of the nature and magnitude of the cancer problem in defined population groups. Since 1956, cancer registries have been an integral part of the American College of Surgeons' effort to improve the quality of care of patients with cancer through hospital-based cancer programs. Connecticut has maintained a statewide cancer registry since 1941 and collected data on cases diagnosed since 1935. Since 1956, the National Cancer Institute has sponsored a collaborative program that brings together data from cancer registries in various parts of the United States. Currently, the program, which is known by the acronym SEER, includes 11 population-based registries with a combined total of 23 million people.

The centralized registries have provided a description of important changes in the cancer picture in the United States during the past 3 decades. The incidence of cancers of the stomach and invasive cancers of the uterine cervix has decreased sharply. The incidence of cancers of the lung, colon, breast, prostate, urinary bladder, and pancreas has increased (*1*, *2*). For several organ sites [breast, uterus (both cervix and corpus), prostate, and bladder], available data (*3*) point to an increase in the proportion of cancers classified as localized when diagnosed and first treated. With the introduction of antibiotics and development of more effective supportive techniques, the proportion of cancer patients treated by surgical resection increased markedly during the late 1940's and 1950's, particularly in the treatment of cancers of the buccal cavity, digestive tract, larynx, and urinary system (*4*). The 1960's were marked by increased use of chemotherapy in the treatment of cancers of the lung, breast, uterine corpus, ovary, prostate, and thyroid, and melanoma of the skin, as well as in the treatment of the leukemias and lymphomas (*4*).

The changes mentioned above were accompanied by encouraging improvement in patient survival rates. Marked increases in survival rates among patients with almost all forms of cancer occurred between the 1940's and 1950's. During the 1960's, improvement in patient survival was less dramatic, but survival rates did increase for forms of cancer that account for more than 40 percent of all malignant neoplasms. Early returns on patients treated during 1970 and 1971 indicate continuing improvement in survival rates for half of all cancers (*5*).

Much of the information made available through continuing, centralized cancer registry programs can be and has been obtained via surveys (*6*, *7*). Continuing reporting systems, however, have several advantages: 1) They provide a mechanism for monitoring patient follow-up and collection of information on therapeutic end results, 2) they facilitate investigation of questions and issues uncovered by routinely collected data through the availability of an experienced technical staff with established working relations in the community, 3) they provide an information system for continuing review of the impact of cancer control programs and the medical care provided to cancer patients, and 4) they make available current information for use in professional and public education programs.

For individual patients, the information collected by hospital registries and centralized registry systems is fundamentally the same. Although specific reporting mechanisms vary, centralized registries essentially are coordinated networks of individual hospital registries. However, when the data pertaining to an individual hospital are interpreted, the following should be considered: 1) Except for a few large specialty centers, the number of patients with a particular form of cancer seen during 1 year, or even 5 years, is small, and observed figures are subject to considerable chance variation; 2) the patients seen in an individual hospital may be drawn largely from a particular segment of the population, and factors not directly related to the disease and the care received in the hospital may influence therapeutic end results; 3) the routinely available data are generally more useful for the formulation of questions to be investigated than for the provision of clean-cut information. This third point is equally applicable to data routinely collected by centralized registry systems.

Medical procedures, both diagnostic and therapeutic, have become more sophisticated and complex during the past 2 or 3 decades. Cancer registries have both an opportunity and an obligation to gear up to provide information relevant to current medical practice. For example, the percentage of cases with microscopically confirmed diagnoses is no longer a useful index of the

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Abbreviation used: SEER=Surveillance, Epidemiology, and End Results.

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quality of diagnostic practice, inasmuch as 90% of cancers are now microscopically confirmed (*6*). Similarly, information on the percentage of surgically resected breast cancers is of limited interest when 90% of all breast cancers are so treated, the extent of the resection is becoming increasingly varied, and the use of adjuvant therapy is becoming more frequent (*8*).

Since January 1976, the criteria for approval of a hospital cancer program promulgated by the American College of Surgeons include "a system for quality-of-care evaluation with documentation of its operation." The NCI-sponsored SEER program provides a model for the collection of descriptive information that is sufficiently detailed for assessment of the quality of medical practice in the care of cancer patients. For example, a site-specific code has been developed for recording pretreatment diagnostic procedures and pretreatment assessment of the extent of disease spread. Thus treatment practice can be assessed in the light of information available before treatment is initiated. Meaningful case review requires examination of the full patient record, but a properly organized cancer registry can be an important instrument in selecting cases for review.

Cancer registries have raised the level of awareness of the medical profession regarding the importance of evaluating the care given to cancer patients. The data produced in past years by both individual hospital and centralized registries have been useful, but have tended to be "crude" (i.e., lacking in detail). Registries can more fully contribute to the continuing improvement of the quality of care given to cancer patients by collecting information in sufficient detail to facilitate review of diagnostic and treatment practice while they continue to promote patient follow-up and provide information on end results.

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GUEST EDITORIAL by **Ernst L. Wynder**, *M.D.* [1] *and* **Gio B. Gori**, *Ph.D.* [2]

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Contribution of the Environment to Cancer Incidence: An Epidemiologic Exercise

Most cancers today appear to be induced by elements originating in man's environment rather than as a result of purely genetic or viral factors. While mechanistic interpretations generated by classic observations on cancer etiology vary, they agree on the extent of environmental influence on the development of certain forms of cancer. During the past two decades epidemiologic studies have elaborated on this concept and suggest that cancer may not be an inevitable consequence of aging.

Doll (*1*) and Higginson (*2*) have argued that the lowest reported cancer rates represent "baseline" or "natural" rates. Generally any increases from these baseline data can be attributed to environmental influences. Based on comparisons of high- and low-incidence ratios in different regions, Higginson (*2*) concluded that 90% of human cancer incidences can be ascribed to environmental factors. Boyland (*3*) claims that this same percentage is due to chemical components and attributes the remaining 10% of cancer incidence to genetic, viral, and radiation factors. We have defined environmental elements influencing cancer incidence as those originating wholly or largely outside the host's body. These include carcinogens, cocarcinogens, promoters, procarcinogens, and other modifying agents. In many instances, an insufficient amount of hard data exists to make definitive statements concerning cancer incidence and causative factors. Therefore, we have attempted to estimate the extent that cancer incidence and mortality may be related to certain suspect causative factors. Hopefully, such an analysis will stimulate further meaningful studies that will permit formulation of more definitive statements on cancer causation.

To determine more precisely the influence of individual causative factors, the evidence related to specific environmental elements has been examined. Differences between population groups, sex ratios, time trends, and demographic and socioeconomic variables have been investigated. We have assumed that environmental factors do not affect all population groups equally. Although no group is untouched by environmental influences, in general the populations with the lowest rates would have the least contact with specific causative factors. Therefore, examination of the extremes of cancer rate differences by site constitutes a plausible basis for inferences on environmental causes.

BASIS FOR ESTIMATES

Intercountry and Intracountry Comparisons

Mortality and incidence rates for major cancer types show significant differences among countries (*4–6*). The distribution of extremely high and low death rates has been calculated. The percent difference between rates for U.S. whites and low-risk countries and the differences between the high- and low-risk countries are presented in tables 1 through 4. These differences represent the percentage of cancers in excess of a baseline or natural rate.

The worldwide distribution of mortality rates for males (table 1) illustrates the environmental influences on all cancer sites. The ratio between high- and low-risk rates is greater than 2.0 for all forms of cancer. Except for leukemia, the same is true for female mortality ratios. The proportion of incidence rates for both men and women related to environmental elements is indicated in tables 3 and 4. Data have been standardized by age, and the observed variations cannot be explained by this variable. Obviously, it is necessary to consider differences in the quality of vital statistics relative to the reportability of the various cancers, the accuracy of population estimates, and the accuracy of disease diagnoses. In some of the less developed countries, for example, it is likely that mortality and incidence data are underestimated; however, data from select cancer registries that may be regarded as more accurate also indicate comparable large differences between high- and low-risk populations. For example, the United States and Japan have high standards for recording vital statistics, and the large variation in incidence rates is apparent between these countries (tables 5, 6).

The effect of environmental factors on the incidence of cancer can also be evaluated by the comparison of rates within a given country. Perhaps the most striking example is the percent excess of skin cancer rates. This disease is related directly to the intensity and length of exposure to sunlight. A similar, though less distinctive, excess difference has been found in the incidence of melanoma between the northern and southern regions of the United States (*8*). This relationship between incidence of melanoma and exposure to sunlight has been demonstrated in Israel as well (*9*).

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A survey by Hoover et al. (*10*) showed differences in rates for various types of cancer among counties within the United States. The cancer rates were higher in certain industrial counties than in nonindustrial centers, especially for bladder and lung cancer. However, standardization of data by smoking habits for some cancers is necessary before etiologic factors can be determined.

Intracountry differences for stomach cancer usually involve higher incidence rates in northern regions than in southern regions. In Yugoslavia, for example, gastric cancer is far more common in the north than in the south (*11*), and similar, though not as extreme, differences exist in the United States (*10*). Reasons for these geographic differences remain unclear but appear related to dietary practices.

Population groups such as Seventh Day Adventists and Mormons provide well-documented evidence of the influence of environmental factors, notably diet and smoking habits, on cancer incidence (*12, 13*). Racial and ethnic differences within a country also provide insights into probable environmental influence on cancer risk. For instance, Jews have a low rate of cancer of the upper alimentary tract, related to their low intake of alcohol. Cervical cancer among this ethnic group is one-tenth that of the general population (*14, 15*). In France, the incidence rate of esophageal cancer is significantly higher in Brittany than in other provinces; this is consistent with data on the intake of alcoholic beverages peculiar to this area. Racial differences are illustrated in the significantly greater incidence of prostate and cervi-

TABLE 1.—*Age-adjusted male mortality ratios for high- and low-risk populations and for U.S. whites by cancer site, 1966–67*[a]

Cancer risk	Low-risk country	U.S. white[b]	High-risk country[c]	
Trachea, bronchus, lung	Portugal	3.6	7.1	Scotland
Stomach	United States (for whites)	—	7.8	Japan
Prostate	Japan	6.9	12.4	United States (for non-whites)
Intestine except rectum	Japan	3.9	4.3	Scotland
Esophagus	Norway	1.3	5.9	France
Larynx	Sweden	3.8	19.8	France
Rectum	Chile	2.4	4.8	Denmark
Buccal cavity, pharynx	Israel	3.1	7.2	France
Pancreas	Italy	1.9	2.3	United States (for non-whites)
Bladder	Japan	2.1	3.3	S. Africa
Skin	Japan	3.4	5.5	Australia
Liver, biliary passage	Norway	1.6	5.3	Japan
Thyroid	New Zealand	1.4	5.5	Switzerland
Leukemia	Japan	2.0	2.1	Denmark

[a] *See* (*5*).

[b] U.S. mortality rate/low-sk country mortality rate.

[c] High-risk country mortality rate/low-risk country mortality rate.

TABLE 2.—*Age-adjusted female mortality ratios for high- and low-risk populations and for U.S. whites by cancer site, 1966–70*[a]

Cancer risk	Low-risk country	U.S. white[b]	High-risk country[b]	
Stomach	United States (for whites)	—	7.8	Japan
Breast	Japan	5.5	6.6	Netherlands
Uterus (all parts)	Israel	1.9	4.7	United States (for non-whites)
Intestine, except rectum	Japan	3.7	4.3	Scotland
Trachea, bronchus, lung	Portugal	2.4	4.2	Scotland
Rectum	Chile	1.6	3.3	Denmark
Pancreas	Italy	1.9	2.4	United States (for non-whites)
Esophagus	Austria	1.0	6.9	Chile
Bladder	Japan	1.5	2.4	United States (for non-whites)
Skin	Japan	2.5	4.2	Australia
Buccal cavity, pharynx	Federal Republic of Germany	2.4	3.8	N. Ireland
Larynx	Norway	4.4	16.8	Ireland
Liver, biliary passage	New Zealand, Federal Republic of Germany	1.6	4.1	Federal Republic of Germany
Ovary, fallopian tube, broad ligament	Japan	3.9	5.9	Denmark
Thyroid	Australia	1.1	4.0	Austria
Leukemia	Japan	1.6	1.9	Israel

[a] *See* (*5*).

[b] *See* footnotes *b* and *c*, table 1.

TABLE 3.—*Age-adjusted incidence ratios for high- and low-risk populations and for U.S. whites by cancer site: Males*[a]

Cancer site	Low-risk country	U.S. white[b]	High-risk country[b]	
Lung, bronchus, trachea	Nigeria	4.7	72.1	United Kingdom
Stomach	Nigeria	9.5	79.4	Japan
Prostate	Israel	12.8	14.0	United States (Hawaii)
Colon	Rhodesia	50.0	60.0	Canada
Bladder	New Zealand	19.7	21.4	United States (Hawaii)
Rectum	Rhodesia	136.0	136.0	Canada
Esophagus	Israel	36.0	361.0	Rhodesia
Pancreas	Nigeria	6.6	14.7	New Zealand
Kidney	Nigeria	7.8	9.4	Sweden
Larynx	S. Africa	16.7	34.5	India
Nasopharynx	S. Africa	7.0	104.0	United States (Hawaii)
Skin, melanoma	Japan	42.0	62.0	New Zealand
Small intestine	Rhodesia	10.0	11.0	Canada
Liver	Poland	23.0	475.0	Rhodesia
Thyroid	S. Africa	20.0	57.0	United States (Hawaii)
Leukemia	Rhodesia	5.2	7.0	United States (Hawaii)
Lip	S. Africa	141.0	286.0	Canada
Tongue	United States (Hawaii)	26.0	140.0	India
Pharynx	Canada	20.0	160.0	India

[a] *See* (*6*) and (*7*), adjusted to standard world population.

[b] *See* footnotes *b* and *c*, table 1.

TABLE 4.—*Age-adjusted incidence ratios for high- and low-risk populations and for U.S. whites by cancer site: Females*[a]

Cancer site	Low-risk country	U.S. white[b]	High-risk country[b]	
Breast	Israel	7.9	7.9	United States (for whites)
Stomach	United States (Hawaii)	3.3	30.4	Japan
Cervix	Israel	5.1	29.6	Rhodesia
Colon	Rhodesia	44.0	55.0	United States (Hawaii)
Lung, bronchus, trachea	Nigeria	12.1	37.7	New Zealand
Uterus	Japan	13.7	18.3	United States (Hawaii)
Rectum	Rhodesia	83.0	136.0	Canada
Bladder	United States (Hawaii)	44.0	569.0	Rhodesia
Skin, melanoma	United States (Hawaii)	40.0	91.0	New Zealand
Kidney	Nigeria	33.0	60.0	Sweden
Pancreas	Nigeria	6.1	15.3	United States (Hawaii)
Esophagus	Israel	11.0	361.0	Rhodesia
Nasopharynx	Colombia (Cali)	2.0	46.0	United States (Hawaii)
Larynx	S. Africa	8.0	28.0	India
Small intestine	Canada	6.0	11.0	Canada
Liver	India	11.0	342.0	Rhodesia
Ovary	Japan	6.4	8.7	United States (Hawaii)
Thyroid	Hungary	7.1	29.5	United States (Hawaii)
Leukemia	S. Africa	9.0	84.8	Rhodesia
Lip	Japan	2.0	19.0	S. Africa
Tongue	Rhodesia	9.0	30.0	S. Africa
Pharynx	Hungary	5.0	44.0	India

[a] *See* (*6*) and (*7*), adjusted to standard world population.

[b] *See* footnotes *b* and *c*, table 1.

TABLE 5.—*Age-adjusted incidence per 100,000 population of selected cancer sites for males in the United States (Connecticut) and Japan (Miyagi)*[a]

Site	United States	Japan	Incidence ratio[b]
Bronchus, trachea	44.0	15.6	2.8
Prostate	33.0	3.2	10.3
Colon	26.7	4.1	6.5
Bladder	19.9	4.7	4.2
Rectum	16.3	4.8	3.4
Stomach	14.7	95.3	0.1
Pancreas	8.1	6.7	1.2
Kidney	7.6	1.8	4.2
Larynx	7.5	2.4	3.1
Esophagus	5.7	14.5	0.3
Mouth	4.0	1.1	3.6

[a] Adjusted to world population. Data from (*6*).

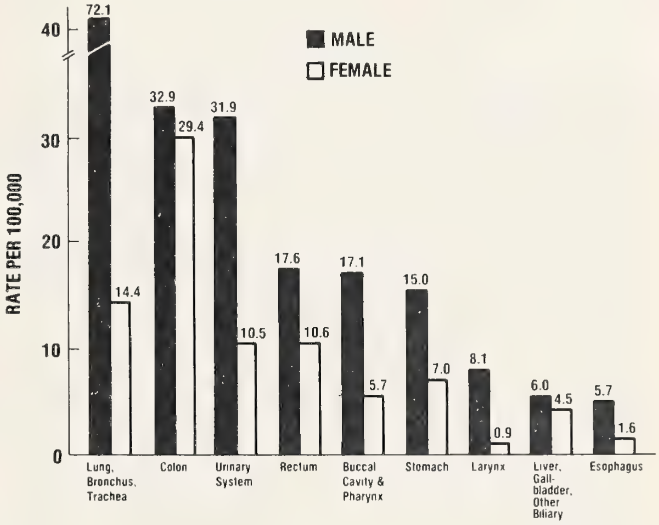
[b] Represents U.S. incidence/Japan incidence.

TABLE 6.—*Age-adjusted incidence per 100,000 population of selected cancer sites in females in the United States (Connecticut) and Japan (Miyagi)*[a]

Site	United States	Japan	Incidence ratio[b]
Breast	62.3	11.0	5.6
Colon	26.7	4.0	6.6
Corpus uteri	15.3	1.3	11.7
Ovary	11.3	1.9	5.9
Rectum	10.7	5.0	2.1
Bronchus, trachea	7.8	6.0	1.3
Stomach	6.8	44.7	0.1
Bladder	5.9	1.6	3.6
Pancreas	4.3	3.8	1.1
Kidney	3.5	0.7	5.0
Esophagus	1.4	4.9	0.2
Mouth	1.4	0.6	2.3
Larynx	0.8	0.3	2.6

[a] Adjusted to world population. Data from (*6*).

[b] Represents U.S. incidence/Japan incidence.



TEXT-FIGURE 1.—Average annual age-adjusted (1970 standard) incidence rates per 100,000 population, by primary site and sex for non-sex-linked cancer sites, in the United States for 1969–71 (*7*).

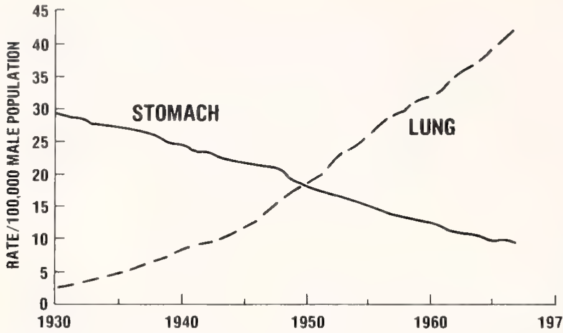
cal cancer observed in blacks as compared to whites in the United States (*8*).

Sex Differences

The effect of environmental factors is also indicated by the different rates for males and females of certain primarily "non-sex-linked" types of cancer (i.e., those not likely to be dependent on specific sex hormones). As expected, these differences are largest among the tobacco-related cancers (text-fig. 1). Some of the observed differences are decreasing, and this trend is expected to continue as women are exposed increasingly to the work, social, and recreational environment of men. For example, three decades ago there was approximately 90% difference in lung cancer rates between males and females. This difference diminished considerably as more women began smoking cigarettes. Thus, as environmental exposure for women increases, similar incidence rates of non-sex-linked cancers can be expected for males and females.

Time Trends

Another useful approach in determining the role of environmental factors in carcinogenesis is the investigation of the change in cancer rates within a specified period of time. Lung and stomach cancer death rates for U.S. men serve as prime examples (text-fig. 2). Since 1930 there has been a steep and steady increase in lung



Text-figure 2.—Male cancer death rates in the United States from 1930 to 1967 (age-adjusted by site).

cancer; this is consistent with increased cigarette smoking in the general population during this period. The sharp decrease in stomach cancer, however, suggests a decrease in some environmental element or the introduction of a dietary protective factor(s).

Case-Control Studies

Retrospective and prospective epidemiologic studies can contribute not only to an understanding of the incidence of cancer in the presence of a given etiologic factor but also to an increased knowledge of the etiology of such cancer. Such studies depend on reliable reporting and registration of data.

When specific etiologic agents are suspected and can be readily identified (e.g., tobacco, alcohol, and industrial materials), case-control studies are of value in estimating death rates in the presence of such agents and in determining their etiologic role.

Retrospective and prospective studies implicating tobacco smoking in the etiology of lung cancer support available vital statistics. Data from these studies indicate that risk for this cancer and cancers of the oral cavity and larynx are low (1–4/100,000) in the absence of smoking (*16–19*). In addition, about 30–50% of the mortality in males from cancer of the esophagus, kidney, bladder, and pancreas has been shown to be related to tobacco usage, especially in the form of cigarettes (*16–23*). The lower rate for these cancers in females is consistent with the patterns of tobacco usage among women.

SELECT ENVIRONMENTAL FACTORS

Tobacco Use

Epidemiologic evidence for the contribution of tobacco usage to development of cancer is overwhelming (*16–23*).

Prospective studies have determined specific rates of lung cancer in smoking and nonsmoking populations (*16*). These studies have shown that the more definitive the diagnosis of lung cancer (especially in terms of squamous or oat cell cancer), the lower the rate of lung cancer in nonsmokers. Hammond (*16*) showed a rate of 3.4/100,000 for nonsmoking males with histologically proven squamous or oat cell lung cancer. Doll and Hill (*17*) showed a death rate among nonsmoking British male physicians of only 0.7/100,000. Prospective studies of the occurrence of histologically established lung cancer in nonsmoking populations is the best way to determine baseline rates of lung cancer among nonsmokers.

Increases in cancer of the lung have been steady since the 1930's, when the mortality rate in the United States was reported to be less than 4/100,000 (text-fig. 2). This correlates with a steady increase in cigarette smoking during this same period. The increases are more evident for males than females, which is consistent with the fact that less smoking is engaged in by women than by men of comparable age.

Lung cancer rates of populations such as the Seventh Day Adventists, who are nearly all nonsmokers, are more than 80% lower than the rates of the U.S. population in general (*24*).

A reduction of risk has also been noted among long-term filter cigarette smokers as compared to nonfilter cigarette smokers (*16*). In addition, smoking cessation has led to a gradual reduction in the rate of lung cancer to the level of a nonsmoker.

Nutrition

An extensive amount of data relating nutrition to cancer was recently presented in (*22*). This relationship is complex and sometimes perplexing to those who view carcinogenesis principally in terms of a "specific carcinogen." We believe that specific carcinogens play a minimal role in the relationship between nutrition and the development of cancer: Rather, nutritional deficiencies and/or excesses are of great importance as sources of procarcinogens and modifying factors.

The evidence linking diet to cancer is obtained by the comparison of nutritional habits and specific cancer incidence rates among different countries or population groups. Comparisons among countries indicate that "overnutrition" is an etiologic factor for certain types of cancer. Such indications are supported by studies which indicate that cancer patterns of migrant populations tend to change and approach the level prevalent in the host country (*23, 24*). Change in risk patterns for gastric cancer has been linked to diets high in carbohydrates and/or nitrate and low in fat and/or fruit and vegetables. In general, the role of nutrition in cancer etiology is strongly supported by the many epidemiologic studies conducted (*25–41*). Present knowledge provides provoking clues implicating such dietary factors as fat and meat intake, excess caloric intake, and nutritional habits that affect the hormonal and metabolic balances. Additional studies are necessary to elucidate the precise role of specific dietary components.

Dietary elements and nonincorporated contaminants are most likely the responsible factors in cancer rates. This view is supported by data from migrant studies (*23, 25*) and from intracountry studies of population groups living in the same environment (*8, 12–15*). Other data have linked a decline in gastric cancer incidence to an improved dietary experience (*24*).

Several examples suggesting diet rather than contaminants as a causative factor are readily brought to mind. For example, air pollution conditions between the United States and Japan are similar; however, the diets are different and, as illustrated in tables 5 and 6, the cancer incidence rates for diet-related sites are dissimilar. The diets of population groups such as Seventh Day Adventist, Jewish, and black populations vary greatly from those of the general U.S. population and these differences are reflected in their cancer rates (*8, 12, 14, 15*). It has been noted that, as countries improve their dietary experience, gastric cancer rates decline (*27*). Smokers inhale excessive amounts of tobacco-type carcinogens, but their risk of diet-related cancers is not significantly different from that of nonsmokers; this suggests that air contaminants are not causative factors for these particular cancers. There are no epidemiologic data that identify food additives or contaminants such as DDT[3] as etiologic factors (*42, 43*). Long-term studies by Armstrong and Doll (*44, 45*) have shown that the rate of bladder cancer in diabetic patients is not higher than rates for the general population.

Air and Water Pollution

Considerable attention has been paid to air pollution as a causative factor in a variety of cancers, such as those of the lung, stomach, and prostate (*46–48*). Studies have concentrated on the effects of pollution through exposure via direct inhalation of polluted air and ingestion of matter deposited on various vegetables and fruits. However, variables such as cigarette smoking and socioeconomic factors tend to complicate an analysis of air pollution and cancer risk.

Fraumeni's study (*49*), which encompassed 44 states, indicated no differences in lung cancer rates when data were standardized for cigarette smoking. Similarly, Buell and Dunn (*46*) found no differences when different levels of air pollution were studied in California. However, the more recent study of Pike et al. (*48*) has linked polycyclic hydrocarbons in air to lung cancer risks.

Hammond's review (*50*) emphasizes the difficulty of accurately measuring the amount and degree of air pollutant exposure encountered by an individual. Hammond has analyzed the American Cancer Society's prospective data on males living 10 years or more in their present area. No significant difference was found in mortality from lung cancer in relation to either residence or size of residential environment. Furthermore, when occupational exposures were excluded and data standardized by age and tobacco consumption, general urban pollution elements were not found to be etiologic factors in lung cancer risks. These data support the earlier work of Doll and Hill (*51*). Further investigation is needed to understand the complex epidemiologic issues involved in air pollution and cancer.

A recent study of the drinking water in New Orleans suggests that chlorinated hydrocarbons could present a potential carcinogenic hazard for man (*52*). Future studies are needed to determine hazardous dose levels of water contaminants.

Occupational Exposure

There is no question that certain occupational exposures increase risk for particular cancers. Specific agents are listed in table 7. Some of these agents, particularly asbestos and uranium dust, exhibit synergistic effects with cigarette smoke, thus increasing a smoker's risk of lung cancer. Both uranium dust and asbestos exposure can lead to cancer incidences in the absence of smoking. Other agents in table 7 are also independently carcinogenic.

Generally, the data reported on occupation-related cancers do not permit annual incidence estimates, since the cases reported usually represent a sample accumulated over several years, rather than those for a specific year. Data on polyvinyl chloride lend themselves more readily to estimates because a rare tumor is involved. Thus far a direct association between exposure to polyvinyl chloride and death from angiosarcoma of the liver has been reported in 38 cases (*53, 54*). The effect of *bis*-chloromethyl ether has been determined on the basis of number and duration of workers exposed, the number of lung cancers reported, and the increase in risk for this cancer among exposed workers compared to the

TABLE 7.—*Occupations conveying an increased risk of developing cancer*[a]

Site	Agents	Occupations
Liver	Arsenic Vinyl chloride	Tanners; smelters; vintners; plastic workers
Nasal cavity, sinuses	Chromium Isopropyl oil Nickel Wood and leather dusts	Glass, pottery, and linoleum workers; battery makers; nickel smelters, mixers, and roasters; electrolysis workers; wood, leather, and shoe workers
Lung	Arsenic Asbestos Chromium Coal products Dusts Iron oxide Mustard gas Nickel Petroleum Ionizing radiation *Bis*-chloromethyl ether	Vintners; miners; asbestos users; textile users; insulation workers; tanners; smelters; glass and pottery workers; coal tar and pitch workers; electrolysis workers; retortmen; radiologists; radium dial painters; chemical workers
Bladder	Coal products Aromatic amines	Asphalt, coal tar, and pitch workers; gas stokers; still cleaners; dyestuff users; rubber workers; textile dyers; paint manufacturers; leather and shoe workers
Bone	Ionizing radiation	Radium dial painters
Bone marrow	Benzene Ionizing radiation	Benzene, explosives, and rubber cement workers; distillers; dye users; painters, radiologists; radium dial painters
Skin	Arsenic Sun	Insecticide makers and sprayers; oil refiners; vintners; smelters; farmers

[a] *See* King H: Cancer risk and life style, presented at the meeting of the American Association for the Advancement of Science, Boston, Massachusetts, in February 1976.

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[3] DDT = 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane.

general population (*55*). Although the increase in risk is considerable, the total number of excess cases is small because of the relatively small number of workers involved.

The data presently available are, at best, educated estimates of the relationship between specific cancers and specific occupational groups. Cole et al. (*56*) suggested that 20% of bladder cancers occurring in males in the Boston area are related to occupational exposure. In certain counties of New Jersey, the increased risk for this cancer appeared to be high among workers in certain chemical industries. Bailar (personal communication) estimated that the occupational contribution to total cancer incidence in males lies between 1 and 5%, and a similar estimate was made by Nelson (personal communication). General estimates of the percentage of all human cancers related to occupational exposure range between 1 and 10%. However, identification of specific high-risk groups, hazardous exposure levels, and related cancer incidence rates is yet to be determined.

Drugs

Various drugs have been linked to cancer risk (*57*), but there are insufficient data to determine the precise relationship involved. It seems clear that the risk of reticulum cell sarcoma is relatively high in patients receiving immunosuppressive drugs, although the total number of cases thus far reported is small (*58*). The early data on reserpine have recently been disputed in terms of the alleged influence of this drug on breast cancer (*59–61*). Premarin appears to increase the risk of cancer of the endometrium, but again the number of cases is in question (*62, 63*). Because of the widespread use of the hormone and reported increases in the rate of this relatively common tumor, careful attention is warranted. With the exception of inducing benign liver tumors (*64, 65*), the use of oral contraceptives is not linked to increased cancer incidence (*66*). Definitive conclusions in this regard, however, should be postponed until the long-term effect can be analyzed.

EPIDEMIOLOGIC CONSIDERATIONS

Mortality and incidence rates in certain low-risk countries may not reflect the true rate for the area. In some of the developing countries, there may be an underreporting of cases because of a lack of sufficient facilities, a lack of standardized definitions of diagnoses, and cultural mores which prohibit attention to specific diseases or parts of the body. Also, migratory populations may not typically reflect the population from which they originated. Although such inaccuracies may exist, particularly in mortality statistics, the consistency with which certain types of cancers appear to be "missing" in particular settings, in conjunction with the verified failure to find suspected carcinogenic stimuli in these areas, gives credence to the reported low rates. These low rates, however, do not preclude the exposure of some individuals to carcinogenic factors, although at lower rates than individuals in high-risk and high-rate countries.

International comparisons of morbidity and mortality rates indicate that most cancers are related to man's lifestyle, including excessive smoking, alcohol consumption, "overnutrition," and industrial exposures. This lifestyle is a relatively recent occurrence and parallels the development of industrial societies. Such phenomena were unknown in more primitive societies and hardly known to animals at all. Although this latter point is often ignored, it is important to recognize that most of the cancers common in man are exceedingly rare in feral animals.

CONCLUDING REMARKS

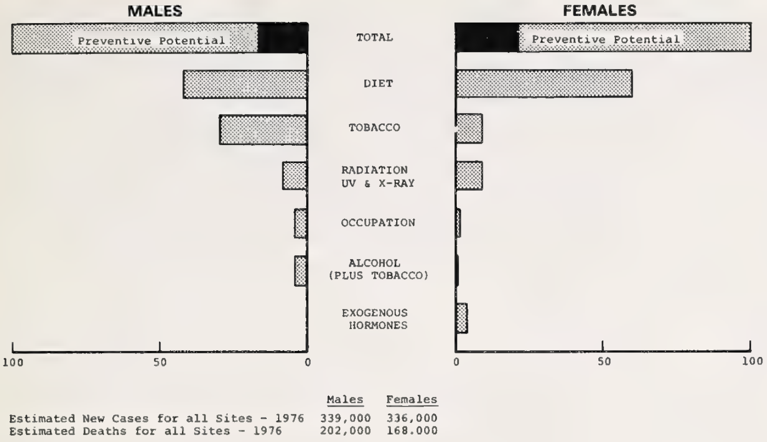
Environmental carcinogens, either established or suspected, may be categorized as being related either to personal lifestyle (tobacco, alcohol, sunlight, occupational exposure, and nutritional imbalances) or to general environmental factors (air and water pollution, drugs, and food additives as contaminants). Some are derived through individual choices; others are imposed by society.

Individuals tend to ignore their own responsibilities and blame harmful occurrences, including carcinogenic exposure, on outside forces. Therefore the general environmental elements receive the most attention rather than factors resulting from personal lifestyles. Individuals cannot deal with some of these issues by themselves; thus public action is required and should be provided. To the extent that industry can reduce carcinogens, cocarcinogens, or procarcinogens (e.g., in the area of tobacco, alcohol, and nutrition) and to the extent that government can control the production or use of products containing such agents (including those of industrial origin), preventive measures should be introduced to protect the individual and society at large.

Personal lifestyle, however, also plays an important role in environmental carcinogenesis. Thus public education campaigns and other services must be provided to help people modify habits that are detrimental to health. Then research and public health efforts must follow to reduce man-made and preventable cancers.

The estimates shown in text-figure 3 represent the relative effects of environmental factors on cancer incidence. We estimated the total percent of site-specific cancer attributable to all environmental factors (depicted as "Preventive Potential") by calculating the percent difference between U.S. mortality rates and the lowest reported worldwide mortality rates for each site and by considering specific case–control studies. Our estimates with regard to such variables as radiation, exogenous hormones, and occupation are based on data in the literature that reflect the increased risk involved with a given exposure and the total number of people exposed.

We hope that text-figure 3 will stimulate research to indicate that a reduction of certain causative variables would indeed result in a decrease in specific cancer incidences. To accomplish this, there must be closer collaboration among epidemiologists throughout the world. With better disease case histories, it should be possible to explain further the vast differences in cancer



TEXT-FIGURE 3.—Percent of cancer incidence in the United States attributable to specific environmental factors.

incidence among countries.

In conclusion, our estimates of the role of environmental factors in cancer incidence concur with earlier data of Doll (*1*), Higginson (*2*), and Boyland (*3*). These estimates are supported by epidemiologic evidence including intercountry and intracountry differences, incidence differences between men and women, and time trends. The data presented here support the basic assumption that cancer is a disease produced by environmental factors. Where disputes may arise, however, is in the relative importance of the general environmental factors versus the personal lifestyle contributions to cancer incidence rates.

This presentation should stimulate investigations of the etiologic aspects of specific environmental factors in total cancer incidence and in the incidence of particular types of cancers. Although it is mandatory that attempts are made to remove every factor in the environment demonstrated to increase the risk of cancer, from both a budgetary and public health point of view, it is equally important that sufficient attention is applied to those elements involved in most cancers. With the increasing number of news reports regarding hazardous components, the average citizen considers himself immersed in an uncontrollable sea of carcinogens. Although society must and can play an important role in the reduction of those environmental factors that contribute to cancer incidence, we have shown that an individual can significantly limit his own risk by appropriately altering his lifestyle.

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GUEST EDITORIAL **Robert W. Miller,** ***M.D.*** [1]

JNCI 60:1195–1203, 1978

Cancer Epidemics in the People's Republic of China [2,3,4]

Epidemiology is concerned largely with defining peculiarities in the occurrence of disease, seeking causes (or risk factors), and preventing disease through interventive measures. The Chinese have some novel thoughts and observations on these matters, particularly with respect to three of the four epidemics of cancers that occur in the People's Republic of China. Because scientific publications in China have been limited since 1966, one must go there to learn of these important developments.

This report concerns such information gained during a trip by a U.S. Cancer Delegation to four cities and two rural areas of China from September 19 to October 12, 1977. Henry S. Kaplan, Professor of Radiotherapy at Stanford University, Stanford, California, was chairman of the delegation, which included 4 therapists, a diagnostic radiologist, a pathologist, an expert in chemical carcinogenesis, and 2 epidemiologists.

FOUR EPIDEMICS OF CANCER

The special excitement of cancer epidemiology in China comes from studies in progress or from potential studies that concern the epidemics of cancer. Three of the four types of cancer occur regionally and are apparently due to environmental exposures. NPC and HCC occur excessively in the South, and EC is found in certain areas of the North. The fourth cancer, trophoblastic neoplasia, occurs excessively throughout China and neighboring countries except Japan. The Chinese regard these cancers as "ethnic" (i.e., genetic) in origin and, therefore, not preventable.

The environmental origins of NPC, HCC, and EC are indicated not only by their regionality but also by their diminished frequencies among Chinese who migrate or whose parents migrated to such nonepidemic areas as the United States. A similar diminished frequency of trophoblastic cancers is seen among Chinese in the United States (*1*), which indicates that these seemingly "ethnic" tumors are environmentally influenced and, therefore, potentially controllable.

Great efforts have been made to find the causes of these cancers when they have occurred excessively elsewhere in the world, with little success so far, as, for example, in studies of EC among the Bantu of the Transkei, South Africa (*2*, *3*) or among residents along the Caspian littoral in Iran (*4*).We expected that similar impasses would be encountered by the Chinese, but they have an unexpected advantage in the study of at least two of these regional cancers.

DOMESTIC ANIMAL MODELS

Snuffling Pigs

Soon after arriving in Canton we traveled by bus about 100 miles, across four rivers by ferry to the (rural) Kaosha Production Brigade in Chung Shan County. There a barefoot doctor (in sandals) told us how the epidemic of NPC affects his particular area. Among 3,000 people in the Brigade, 6 have developed the tumor in the past decade. We watched as he skillfully performed a screening examination (nasopharyngoscopy) on a patient. We saw 1 woman with NPC at 23 years of age; this finding indicated that the exposure responsible may well have been in childhood.

The concentration of cases in this small community, epidemiologically similar to the cluster of diethylstilbestrol-induced vaginal cancers in Boston, Massachusetts, led several of us to think that if only we could spend a few days in the area, we might be able to find the cause. Little did we realize that by returning to Canton we would learn of an important clue to etiology that came from elsewhere in Kwangtung Province.

At the Tumor Institute in Canton, during an introductory overview, 2 pigs with NPC were mentioned.

ABBREVIATIONS USED: EC = esophageal carcinoma; HCC = hepatocellular carcinoma; NPC = nasopharyngeal cancer.

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[4] The author's views do not necessarily reflect those of the U.S. Cancer Delegation, whose report will be published by the National Academy of Sciences.

Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

Later, as we toured the laboratories, we were shown the heads of 2 pigs that were cut in sagittal sections and preserved in specimen jars. One pig had NPC and the other had a carcinoma of the maxillary sinus. We pursued the matter and learned that the finding of several such pigs had drawn attention to an area in Kwangtung Province where four breeding farms within 50 km of one another had a cluster of affected animals. Among 800 female pigs, 2 were found with NPC and 10 others had cancers of the maxillary sinus or nasal passages—quite close anatomically to the nasopharynx. Thus one might regard this cluster as having an etiology in common. Eleven other pigs in the survey were said to have had nasopharyngeal hyperplasia, a possible precursor of NPC observed also in man. These findings, described to us by Dr. Ch'en Chien-ching, are to be prepared for publication in a Chinese journal, the English name of which is *Research on Cancer Treatment and Control*.

If the disease in pigs is indeed a domestic animal counterpart of the human tumor, the search for a cause in man can be narrowed to the small environment of the pig as contrasted with the diverse exposures of the human.

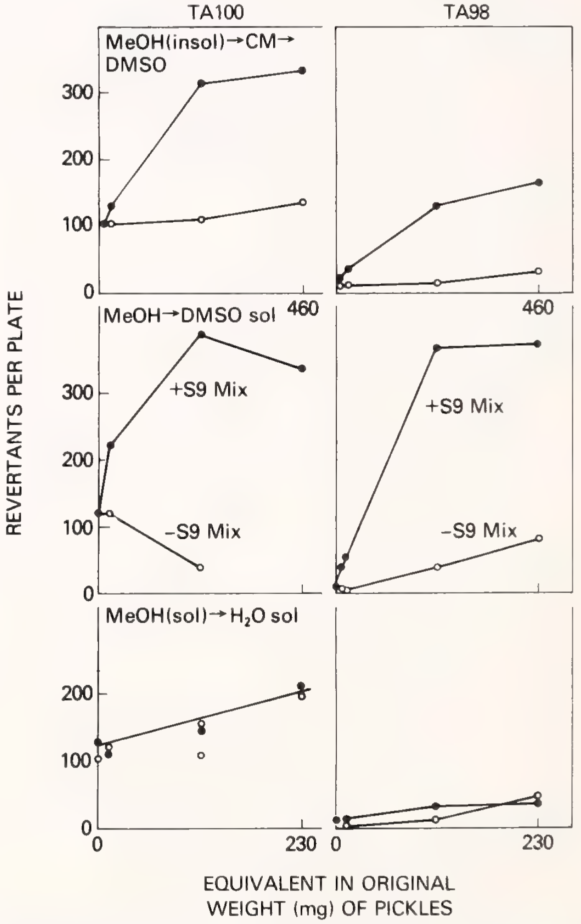
Dysphagic Chickens

We traveled 6 hours by train and 2 by bus to Linhsien County, which is about midway between Peking and Shanghai but further inland. There a dramatic parallel has been observed in the high frequencies of EC in humans and gullet (mainly pharyngeal) carcinoma in chickens. Among the 720,000 persons in the county, one new case of EC is described every 8 hours, on the average. This rate is 50 times greater than that for U.S. whites and equal to the incidence for all cancers in Shanghai.

An excess of gullet cancers among chickens in the county was first described by the Chinese at the Eleventh International Cancer Congress in Italy in 1974 and later published (*5*). Since then, a study of the age distribution of 152 affected chickens that were brought in from remote areas by mailmen has shown that the youngest were 2–4 years of age, with a peak occurrence at 5–6 years (*6*). The environment of the chicken is readily manipulated, thus simplifying the search for the carcinogen(s) responsible. If, for example, 3,000 chickens from high-risk flocks were fed grain instead of table scraps, the absence of cancer of the gullet in 3 years would indicate that the carcinogen indeed was in the food eaten both by the people and the chickens. Should the avian cancer be a counterpart of human EC, but with a much shorter latent period, changes in the rates among chickens, for whatever reason, would foretell changes in the rates for man.

Of even more compelling interest, the search may be further narrowed by the findings of an unusual natural experiment (*7*). More than 50,000 people of Linhsien County had to be relocated when a reservoir was built at the site of their homes. They moved 360 miles to Chung-hsiang County (Hubei Province), where the frequency of EC is low. They are said to have brought only 1 chicken with them and to have acquired new ones from local flocks free of gullet cancer. That was in 1967–68. Since then, 12 of 5,484 chickens have developed cancer, as compared with none among 2,371 chickens of similar age distribution owned by natives of the area. Five of the 12 gullet cancers were associated with EC in the owners or their neighbors. Although the water, air, and soil of Linhsien County were left behind, the carcinogenic effect in chickens arose anew. Thus the carcinogen moved with the people, perhaps in the recipes they use for preparing foods, the scraps of which are fed to the chickens. A leading suspect is a pickled vegetable mix, a specialty of Linhsien County. The Chinese gave 2 of us samples to be tested for carcinogens, and they were positive in the Ames system (text-fig. 1).

Among the people and chickens still in Linhsien County, no change in esophageal/gullet cancer rates occurred after the opening in 1969 of the Red Flag Canal, an extensive irrigation project that vastly altered



TEXT-FIGURE 1.—Bacterial mutagenicity of Linhsien pickle-mix in two strains of *Salmonella typhimurium* (Ames test), as performed by Dr. M. Nagao in the laboratory of Dr. T. Sugimura, National Cancer Center Research Institute, Tokyo. MeOH=methanol; CM=chloroform-methanol mixture (1:1); DMSO=dimethyl sulfoxide.

the ecology of the previously arid county. In a small group of people who migrated to low-rate areas up to 100 years ago, nine cases of EC were said to be known among them or their descendants. Again, adherence to a long-retained special way of preparing a food could be the explanation.

Cirrhotic Ducks

The third such parallel between a domestic animal and man was the least well developed: HCC in a few cirrhotic ducks near Shanghai, where the neoplasm occurs excessively in man. Unlike NPC in pigs and gullet cancer in chickens, no systematic survey of ducks for HCC had apparently been made. The Chinese have suggested that aflatoxin was responsible, because, under experimental conditions, 7-day-old ducks were especially susceptible to aflatoxin-induced HCC (*8*). The study by Carnaghan (*8*) has never been replicated, and there are no reports of ducks on farms being so affected.

NPC, HCC, and EC are extremely rare in U.S. domestic animals, as revealed by a wide surveillance principally of dogs, cats, horses, and cattle [(*9*, *10*); Priester WA: Unpublished data]. Perhaps domestic animal models are found in China because the specific tumors are regionally epidemic, a consequence of a powerful carcinogen, and because the animals are kept for their full life-spans for breeding or egg laying. The pigs in Canton, used for breeding, were 3–12 years old. In Linhsien County, the people do not like to eat chicken; they keep their flocks mainly for the eggs. Also, the epidemics of human cancer are so well known through public health education that people are alert to the corresponding signs in their animals. Discovery of gullet cancer in chickens was made when a barefoot doctor asked a peasant if he had signs of esophageal cancer. He said he did not but his chicken did.

FOURTH EPIDEMIC: TROPHOBLASTIC CANCERS

At several hospitals we saw small groups of young women with choriocarcinoma or, less frequently, malignant mole. At the Capital Hospital in Peking 26 women on the wards were being treated for these tumors. The numbers of beds allotted for patients with trophoblastic tumors have been reduced from 45 to 28 because the disease, except when it is widespread, can be successfully treated in less specialized centers. This formerly lethal cancer is now almost always curable by chemotherapy. The cure, however, has directed attention away from the cause.

Recently a benign form of trophoblastic tumors, classical (complete) hydatidiform mole, in women of Spanish or Japanese origin has been found to consist of cells whose chromosome markers indicate that they were derived entirely from the father instead of equally from each parent (*11*). The question now is: What environmental factors predispose to this aberration in human reproduction? The mystery may be solved in part in a high-risk country such as China.

A PREDICTABLE FIFTH EPIDEMIC?

In public, the Chinese as a rule express the belief that cigarette smoking is not a cause of lung cancer. In a small group discussion with us, epidemiologists on the faculty of the First Shanghai Medical College argued strongly that the small increase in relative risk of lung cancer among smokers in China (1.7 vs. 10 in the United States) is attributable to chronic bronchitis. Members of the U.S. Cancer Delegation predicted an epidemic when enough people, who can now afford cigarettes, smoke for a sufficiently long time. A report from Hong Kong earlier this year, however, describes a proportionate increase in the main histologic types of lung cancer in men and women in Hong Kong, instead of a disproportionate increase in Kreyberg Group I tumors as expected from cigarette smoking (*12*). This observation reopens the question of the susceptibility of Chinese to lung cancer from cigarette smoking. Another possibility is that Chinese tobacco is noncarcinogenic; i.e., the Chinese have happened upon a safe cigarette. Two small packs of cigarettes (different brands) given to us on air flights within China have since been studied to determine if the bacterial mutagenicity of the smoke as measured by the Ames test is comparable to that of cigarettes from the United States, Europe, and Japan (*13*). The mutagenicity was similar to that for Japanese cigarettes (Sugimura T: Personal communication).

OCCUPATIONAL CANCERS

We asked repeatedly about occupational cancers in China, and, at first, were told there were none. Gradually, as the trip progressed, we learned of some mines in which the presence of arsenic and/or radon may explain the excess of lung cancers. We visited a steel mill near Peking where, as in the United States (*14*), coke oven workers have an increased frequency of lung cancer. In Shanghai we were told that "NC," a white powder used as an antioxidant in the rubber industry, is carcinogenic in animals and may have something to do with an excess of lung cancer among workers in one of several factories in which the compound is used.

We asked repeatedly about mesothelioma and were told that it was rare and not associated with exposure to asbestos. Even at the Shanghai Chest Hospital, no asbestos-related mesotheliomas had been seen. In a personal series of 525 soft-tissue cancers described by Dr. Li Yueh-yun, chief of surgery at the Shanghai Tumor Hospital, 48 were mesotheliomas. None was attributed to asbestos.

We asked repeatedly about cancer of the urinary bladder, a target organ for occupational carcinogens, and were told that the tumor was rare and had not been linked to industrial exposures, including industries in which dyes were used. Premier Chou En-lai's death from this cancer has not focused attention on it.

We devoted a $2^1/_2$-hour small group discussion one evening to reviewing a checklist of known human carcinogens and learned of none that has been a problem in China. Earlier during our trip, one elderly, wise physician had said that as industrialization of China increases, cancers will occur in addition to acute toxicity already seen. He also told us that environmental health agencies had been created in Shanghai and Peking. Confirmation came during our stay in Peking, when the departure of members of such a unit for a meeting in the Federal Republic of Germany was reported in the English news summary (Hsinhua News Agency) distributed daily at our hotel.

RADIOGENIC CANCER

Cancers of the skin, including melanoma, were said to be rare in China, even in sun-drenched areas. Mortality rates for these cancers in the United States were about the same for Chinese-Americans as for whites (*1*).

We did not ask about tumors from excessive exposure to ionizing radiation because the possibility of such occurrences seemed remote. We did ask about population subgroups with high frequencies of specific genetic disorders that have increased sensitivity to radiation (e.g., xeroderma pigmentosum or ataxia-telangiectasia), but our respondents knew of none. Such subgroups elsewhere in the world are rare but, when recognized and appropriately studied, have yielded important knowledge about the interaction of genetics and the environment at the molecular level (*15*).

PARASITES

Parasitic infestation of the bile ducts by *Clonorchis sinensis* is associated with an increased frequency of cancer at this ordinarily rare site. We were told that in a few parts of China such cancers are still being seen excessively in patients with the infestation.

In 1975 an American Schistosomiasis Delegation visited China and was told that the parasite no longer existed there (*16*). A change in policy was clear when we were told by several people that heavy infestation of the rectum with *Schistosoma japonicum* apparently is related to carcinoma at that site. Heavy infestation of the rectal wall is associated with granulomas that seem to progress to pseudopolyps, true polyps, and finally to carcinoma (*17*, *18*). If confirmed, this would be the third parasite implicated in human carcinogenesis; the other two are *C. sinensis*, already mentioned, and *S. haematobium*, which is associated with a high frequency of cancer of the urinary bladder, particularly in Egypt (*19*).

MAPPING CANCER MORTALITY

A major discovery during our trip was that while staff members of the National Cancer Institute were preparing maps of site-specific cancer mortality, according to county, in a search for high-rate areas (*20*), the Chinese were independently making similar maps in several provinces according to commune. Each commune has 8,000–100,000 people. Six site-specific maps for Kiangsu Province have been printed but will not be ready for publication until the methods and data have been rechecked. With the use of mortality rates for 1973–75, maps have been made for cancers of the lung, liver, stomach, and uterine cervix, leukemia, and all cancers combined. The range of rates for each cancer was divided into six equal intervals. A different color was used for each map, with a spectrum of hues from the lightest for communes with the lowest rates to the deepest for communes with the highest rates. Geologists and geographers, among others, are collaborating in this work. They asked us if we recommended using isopleths based on geographic characteristics, instead of communes, to enlarge the populations studied and to give more meaning to the interpretation of the data. We said that for cancers of low frequency we used State Economic Areas—already established combinations of counties with similar economic conditions. The combining of communes with respect to certain attributes seemed innovative and useful to us.

Maps for six more provinces are expected to be completed soon and eventually may be extended nationwide to all 29 provinces in which there are about 75,000 communes. The map of Kiangsu Province had also been prepared by county, but these geographic areas were too large for the purpose. In one such map a "hot spot" of high-rate communes at the juncture of three counties was washed out when counties were the units studied.

The maps were based on death certificate diagnoses, the vast majority of which were made at county hospitals. The diagnoses were based on pathology reports in 15%, on laboratory tests in 65%, on symptoms in 11%, and on medical history in 4%.

As in the U.S. maps, the map for Kiangsu Province showed a nonrandom distribution of lung cancer, which suggests environmental carcinogens in some locales other than urban areas. With respect to liver cancer, the northern border of the epidemic area was clearly demarcated. Deaths from cancer of the uterine cervix occurred at higher rates than usual in a cluster of communes beyond the reach of the vigorous, Shanghai vaginal cytology screening program.

Research workers in both countries were independently making the same studies and discovering peculiarities of cancer distribution that reinforce one another, but they were unaware of the activities in the other country. This East–West independence in discoveries has, of course, occurred in many other fields for millenia.

GENETIC OR FAMILIAL CANCERS

Studying the inheritance of cancer understandably does not have a high priority in an overpopulated country where masses of people are urged to participate in cancer prevention, detection, and treatment. However, various largely unrecorded observations are of genetic interest.

Esophageal Cancer

EC is one of the rarest tumors to affect close relatives except in the heritable disorder tylosis. Yet in Linhsien County, three branches of one family each had 5–7 members affected. Women who married into these families were at times affected, which suggests the influence of a strong carcinogen in a family whose resistance is low, perhaps for genetic reasons.

During a small group meeting at the Linhsien Hospital on our penultimate evening in China, our hosts generously let me copy parts of a pedigree from an immense scroll for the entire kindred insofar as it was known. Information on female members of the families tended to be unavailable for past generations unless the women were known to have had EC. As the session ended, I realized that another scroll was at hand: the pedigree of a different family in which two branches were heavily affected. One involved several women who had married ~~into~~ the ~~family, and~~ the other included a group of brothers and their sons who had developed the cancer. The cars were waiting to take us back to the hotel, and it was too late to copy these interesting pedigrees, which the Chinese apparently do not plan to publish. They have, however, published a table in which these families, among others, were listed in the proceedings of a meeting, the title of which was translated: *Selected Essays from the Fifth Conference on Prevention and Research Related to Esophageal Cancer in Linhsien County*, issued in February 1972 by the Office on Prevention and the Study Committee for Esophageal Cancer in Linhsien County. The key portion of the table, too long to be reproduced here, will appear in the report of our delegation (*21*).

The histopathology of EC is the same as that seen elsewhere in the world (*21*), but the natural history of the tumor is not. It concentrates especially in the middle third of the esophagus, is associated with multiple foci of in situ tumors, and has a high 5-year survival rate after surgery (≈44% for all stages combined), and patients who refuse therapy sometimes experience spontaneous regression of the tumor.

Nasopharyngeal Carcinoma

In Canton, Dr. Li Chen-ch'uan said that at our last session he would show us pedigrees of two families in which NPC aggregated, but unfortunately a change in his schedule prevented us from seeing them.

Leukemia

As in the Japanese and Singapore Chinese, chronic lymphocytic leukemia is nearly absent in China. Only 1.5% of adult leukemia is of this type, as compared with 30% in the United States (*22*). The absence of a rise in rates (i.e., a migrant effect) among persons of Chinese or Japanese descent in Hawaii indicates a genetic resistance to this form of leukemia (*23*).

In Soochow we learned that among 173 children under 15 years of age in 1973–75, all with bone marrow confirmation of leukemia at the Children's Hospital, about 50% had acute lymphocytic leukemia, as compared with 80% in the United States (*24*). This deficiency suggests an absence of the peak in occurrence of acute lymphocytic leukemia at 4 years of age, seen in U.S. whites but not in blacks, who are apparently not susceptible to the agent responsible for the peak (*25*).

Pinealoma

Because pinealoma occurs about 12 times more often in Japan than in Western countries (*25*), we tried several times to learn its frequency in China. Only the radiotherapist in Canton had data on it. He told us from memory that he had seen (an unremarkable) 11 cases between 1950 and 1975.

Retinoblastoma

Retinoblastoma has a hereditary form that is usually bilateral (25% of retinoblastoma in the United States), whereas nonfamilial cases are usually unilateral. In the United States, Wilms' tumor is twice as common as retinoblastoma, and worldwide, Wilms' tumor seems to have the most constant rate of occurrence of all childhood tumors (*25*). We were told that the usual ratio was reversed in Canton and Shanghai and that retinoblastoma was three to four times more common than Wilms' tumor. In about 15% of the patients, both eyes were affected. An excess of retinoblastoma, if well documented in China, would be of great interest because the tumor appears also to occur excessively in other underdeveloped areas—Central Africa, India, and South America (*25*). One would expect the heritable form of the disease, an autosomal dominant trait, to exhibit essentially no geographic variation, so the increase would be expected only in unilateral sporadic cases.

MASS SURVEYS FOR CANCER DETECTION

According to Mao's teaching, in medicine as in other fields, emphasis should be placed on activities for the masses, especially peasants, with special attention to prevention of disease. The Great Wall represents one such mass activity of 2,000 years ago. The building of the 1,500-km Red Flag Canal in 1960–69 in Linhsien County is a recent example. Thus mass surveys for health come naturally to the Chinese, and they undertake such screenings on large numbers of people.

In southern China, in 1970–72, nasopharyngoscopies were performed on 436,786 people by 600 barefoot doctors. The proportion of stage I–II NPC rose from 24 to 87% when found by this method. Some patients with nasopharyngeal hyperplasia were identified. A few cases progressed to NPC, but most regressed.

In Shanghai, 690,000 vaginal smears among textile workers since 1958 have revealed 502 cancers of the uterine cervix; most of them were in early stages. Death from this form of cancer among textile workers has been virtually eliminated.

A survey of serum alpha-fetoprotein levels among 417,644 persons in the Shanghai area revealed 57 with elevated values. In another study of 51 such persons, 15 developed hepatocellular carcinoma within 1$^1/_2$ years, 18 others had persistence of high levels without detectable tumors, and an equal number reverted to normal. When HCC is found by this screening procedure, the tumor is generally small and resectable. In another study, a sample of patients with elevated alpha-fetoprotein levels were prophylactically treated with 5-fluorouracil, and fewer of them developed HCC than did a group treated with protein hydrolysate and vitamins.

In a screening program in Linhsien County, a gauze-covered balloon at the end of gastric tube is passed into the patient's esophagus by a barefoot doctor, inflated, and withdrawn. Esophageal cells on the gauze are examined microscopically. This procedure has been performed about 100,000 times and is said to increase greatly the proportion of cases detected early, when they are readily resected.

FOLLOW-UP STUDIES

In Shanghai there appeared to be no problem in follow up of patients with elevated blood levels of alpha-fetoprotein. In Peking we learned of some difficulty in the determination of the health status of esophageal cancer patients who lived elsewhere. Follow up would seem to be easy because everyone is registered with the local authorities in his or her home area, but apparently the cooperation of these officials is not easily secured.

CITIES AND THEIR CANCER SITE-SPECIFIC STUDIES

Each city we visited focuses its studies on a particular cancer. Canton has, in essence, a task force to study NPC but no emphasis is placed on HCC, which is as frequent as NPC. Shanghai specializes in the study of HCC but not in the study of the more frequent stomach cancer. Peking focuses on EC, whereas Soochow concentrates on leukemia.

The site specificity of the research programs of Canton, Shanghai, and Peking was established after the International Cancer Congress in 1974, in which the Chinese participated. About 10 other centers are apparently in various stages of development in areas where rates for specific neoplasms are high.

CANCER REGISTRIES

Before we left the United States, we had been told by previous U.S. visitors to China that no tabulations of cancer incidence or mortality were available. In Canton we initially tried to obtain information about cancer frequencies from physicians, such as the chemotherapist or radiotherapist, who saw the greatest arrays of cancer patients.

On our first full day in China, during a presentation at the Chung Shan County Hospital, average annual age-adjusted cancer mortality rates for the county, 1970–75, were read to us (tables 1, 2). We did not realize then that the rates for cancers of the stomach and lung were exceptionally low, as compared with data on Chinese elsewhere. But even this short list was more than we had been led to expect.

TABLE 1.—*Age-adjusted cancer mortality rates (per 100,000) for cancer of selected sites and types among males in Chung Shan County, Kwangtung Province, 1970–75; Shanghai City, 1975; Chinese in the United States, 1950–69; and U.S. whites, 1975* [a]

Site or type	Mortality rates per 100,000			
	Chung Shan County [b]	Shanghai City [b]	Chinese in the United States	Whites in the United States
Liver	19.60	22.65	13.02	1.37
Nasopharynx	17.48	—	10.08	0.37
Stomach	6.39	29.39	17.28	8.11
Lung	5.56 [c]	24.40	43.55	61.44
Colon	4.02	2.95	18.41	18.46
Leukemia	3.09	2.93	5.68	8.49
Esophagus	2.33	14.41	8.30	4.23
Rectum	—	2.73	9.14	5.35
Pancreas	—	2.41	8.86	10.25
Lymphoma	—	2.05	7.07	7.11
Urinary bladder	—	1.99	3.18	6.75
Other	—	15.12	45.15	60.59
Total	—	121.03	189.72	192.40

[a] Data from Chung Shan County were read to us. Those from the Shanghai City Tumor Registry, adjusted to a standard world population (*26*), are from charts that we photographed with permission. The U.S. data for Chinese in the United States, age-adjusted to the U.S. population in 1960, are from (*1*). Data (unpublished) on U.S. whites are from death certificates and are age adjusted to the same population.

[b] *Dash* indicates data not presented.

[c] Rates for lung cancer may be in error, as indicated by the corresponding data for Cantonese that live in Hong Kong (*12*).

TABLE 2.—*Age-adjusted cancer mortality rates (per 100,000) for cancer of selected sites and types among females in Chung Shan County, Kwangtung Province, 1970–75; Shanghai City, 1975; Chinese in the United States, 1950–69; and U.S. whites, 1975* [a]

Site or type	Mortality rates per 100,000			
	Chung Shan County [b]	Shanghai City [b]	Chinese in the United States	Whites in the United States
Liver	5.38	8.88	2.94	0.63
Nasopharynx	7.44	—	2.65	0.15
Stomach	3.84	15.88	7.42	4.03
Lung	3.70 [c]	12.41	13.36	14.91
Colon	3.95	3.19	8.74	15.19
Cervix and uterus	6.59	6.39	8.01	7.85
Breast	5.38	5.64	10.09	25.91
Leukemia	2.10	3.44	5.68	5.03
Esophagus	1.21	7.35	2.78	1.14
Rectum	—	3.22	3.03	3.17
Pancreas	—	2.81	3.59	6.27
Lymphoma	—	—	5.91	4.66
Urinary bladder	—	—	1.61	1.81
Other	—	17.35	15.60	34.93
Total	—	85.56	91.41	125.68

[a] *See* footnote *a* for table 1.

[b] *See* footnote *b*, table 1.

[c] *See* footnote *c*, table 1.

Two days later, in Canton, another speaker said that during 1976 the death certificates for the previous 4 years had been reviewed for Kwangtung Province (population, 50 million) and data on cancer had been abstracted. The information on about 6,000 death certificates a year was obtained in 3–4 months.

During our first few days in Shanghai, there was some confusion about the existence of registries there. Then, midst a considerable stir of anticipation among the visitors, charts were posted at the front of our lecture hall that gave incidence and mortality rates for the 10 most frequent cancers (tables 1–3).

Patients with newly diagnosed cancer have been registered in Shanghai City (present population, 5.5 million) since 1960, and death certificate diagnoses of cancer in Shanghai have been registered since 1963. Much, if not all the tabulating was by hand, so only data for 1975 have been analyzed in the short time since policy changes have permitted this activity. Had we visited a year earlier, no tabulated data would have been available. The statistics group in Shanghai hopes to tabulate data for years past and to publish them, perhaps next year, but the clerical task seemed formidable to us. Additional incidence data for 1975 were read to us at a small group discussion on the following day (table 3).

Time did not allow us to go into details about ascertainment of cases. We did learn that names are coded by the number of strokes in the calligraphy for them, and that despite the limited number of surnames, separation is almost always possible by the use of given names. The eighth revision of the International Classification of Diseases (*28*) was used to code hospital diagnoses of cancer by anatomic site. Histologic diagnoses are not yet recorded on the registration forms. Records of deaths from cancer were routinely linked with those for incidence.

In Peking we gathered no data on cancer incidence or mortality except for EC. Mortality data undoubtedly exist for Kiangsu Province, 1973–75, as revealed by the cancer maps described above, and for the six other provinces where the maps are in preparation. Clearly, there is a movement toward coalition of these resources, with the hope that eventually national data on cancer mortality will be available.

We were shown listings of cancer cases at the Kaosha Production Brigade near Canton and at commune and county hospitals. As this practice spreads, collection of cancer mortality data for each province should become easy.

Meanwhile, we can compare the data given to us on cancer death rates in Shanghai with those for Chinese and whites in the United States from 1950 to 1969 (tables 1, 2). Several features are of interest. The rates for cancer of the liver, nasopharynx, esophagus, and stomach were lower in Chinese living in the United States than among those in Shanghai but were substantially higher than those for U.S. whites. Thus environmental influences in the development of these tumors appear to lessen with migration. The remaining excessive cases among Chinese in the United States as compared with those among U.S. whites may be due at least in part to exposures of the Chinese early in life, before they migrated to the United States.

TABLE 3.—*Incidence per 100,000 of the 10 most frequent cancers and cancers of other selected sites and types by sex in Shanghai City, 1975, as compared with that for U.S. whites in 1969–71* [a]

Site or type	Incidence per 100,000 [b]			
	Residents of Shanghai City		U.S. whites	
	Males	Females	Males	Females
Stomach	31.69	15.26	10.5	5.0
Lung	26.72	12.51	57.0	12.1
Cervix and uterus	—	22.45	—	33.8
Liver	20.62	6.81	2.3	1.1
Breast	—	15.16	0.6	64.6
Esophagus	12.67	5.51	3.6	1.1
Thyroid gland	2.37	6.31	2.0	5.0
Rectum [c]	5.35	4.37	13.6	8.3
Colon	4.22	4.44	25.0	22.2
Leukemia	3.93	3.74	10.5	6.3
Nasopharynx	3.23	—	0.7	0.3
Urinary bladder	3.04	1.03	17.8	4.7
Larynx	3.07	0.47	6.7	0.8
Ovary	—	4.67	—	12.3
Testis	0.88	—	3.4	—
Skin, not melanoma	2.19	1.63	—	—
Melanoma	0.53	0.40	4.2	4.0
Kidney	1.80	1.05	7.1	3.4
Hodgkin's disease	1.09	0.88	3.8	2.5
Multiple myeloma.	0.49	0.14	3.0	2.1
Prostate gland	1.02	—	39.7	—
Eye and orbit	0.18	0.11	0.8	0.8
Other	17.85	21.88	54.5	35.6
Total	142.94	128.82	266.8	226.0

[a] Data from the Shanghai City Tumor Registry at *top half* of the table (*above the line*) are from charts that we photographed with permission. Data in the *lower half* were read to us during a small group discussion on September 30, 1977. Shanghai data were age adjusted to a standard world population (*26*). The U.S. data are from the Third National Cancer Survey (*27*) and were age adjusted to the same standard world population (*26*).

[b] *See* footnote *b*, table 1.

[c] Includes rectosigmoid junction.

The opposite pattern was seen for several cancers. Among Chinese males, the lung cancer rate was greater in the United States than in Shanghai but was considerably less than that for U.S. white males. The rates for cancer of the lung in females, however, showed little difference among the three groups, despite the component due to cigarette smoking by U.S. white women. Studies of lung cancer among Chinese women in Hong Kong (*12*) and Singapore (*29*) have revealed the proportion with adenocarcinoma to be unusually high as compared with that of other ethnic groups.

Colon and breast cancer mortality rates were higher among Chinese females in the United States than in Shanghai but still well below the rates for U.S. white females. For males, however, mortality from colon cancer among Chinese-Americans was about equal to that among whites. The reason for the higher rate for Chinese-American males than for females is a puzzle, for no such disparity by sex was found among U.S.

whites or blacks. Japanese migrants to the United States exhibited a similar but less marked increase in rates that appeared first and was more pronounced in males than in females (*30*). If the sex difference in rates for Chinese-Americans was not due to underdiagnosis in females, one must conclude that males were more exposed and/or more susceptible to a carcinogen in their new environment than females were.

Leukemia is more frequently listed on death certificates for Chinese in the United States than for those in Shanghai. The rates were lower than those for U.S. whites, in part because of the near absence of chronic lymphocytic leukemia in Chinese, whether they have migrated or not. The lymphoma mortality rates for Chinese in the United States exceed the rates in Shanghai and are now equal to those for U.S. whites.

Urinary bladder cancer, of special interest because environmental carcinogens may often be involved in its genesis in Western countries (*31*), has a relatively low mortality rate in China and among Chinese in the United States. The difference in incidence between Shanghai males and U.S. white males is even more pronounced: 3.04 versus 17.8 per 100,000 per year (table 3).

The total mortality from cancer per 100,000 females was similar for Chinese women in Shanghai and in the United States but was substantially less than that for U.S. whites. For males, the rates were essentially the same for Chinese and whites in the United States and were significantly higher than those for men in Shanghai. This higher rate in males is presumably due, at least in part, to industrial exposures.

The data on cancer incidence per 100,000 (table 3) show that, among the rarer neoplasms in Shanghai, cancer of the prostate gland stands out because its rate, 1.02, is much less than the rate for U.S. whites, 39.7. Underascertainment may account for a small portion of this difference. The reality of the difference is supported by data on deaths, which show the rate among U.S. Chinese to be 70% below that for whites (*1*). Underascertainment probably accounts for a portion of the very low incidence for all cancers in Shanghai as compared with that for U.S. whites.

These demographic data should provide ideas for a variety of studies in China. Mapping calls attention to local "hot spots," where retrospective studies of occupational and other environmental exposures may generate hypotheses about cause. Only the most frequent cancers can be mapped according to commune, however, because of the relatively small size of the populations at risk in each. As the mapping is extended, it should be possible to determine if the high rates for stomach cancer in Shanghai are found elsewhere in China. The special regional styles of cooking could well affect the frequencies of certain cancers.

For the less frequent cancers, lists, as in tables 1–3, for various more populous areas can reveal differences of potential etiologic importance. It would be interesting to know if cancers with low frequencies in Shanghai (breast, testis, urinary bladder, ovary, and prostate gland) occur with high frequency elsewhere in China, for environmental or ethnic reasons. Some differences have been reported, for example, in the histologic types of lung cancer with respect to major dialect groups of Chinese in Singapore (*29*). The opportunities for such studies in China itself should be exceptional.

The only general description of the age distribution of cancer we received was in Shanghai. The relative frequency of new cases there, as determined by the City Tumor Registry for 1975, is shown with comparable data for the United States, 1969–71, in table 4. The two sets of data differ markedly for all age groups except for children under 15 years. Among young adults in Shanghai, cancers of the stomach, thyroid gland and rectum accounted for a surprisingly high proportion of all malignant neoplasms. Among persons about 45 years of age, cancer of the stomach and lung accounted for about 40% of all malignant neoplasms. These sites are obviously important for cancer control in the future.

INTERVENTIVE EPIDEMIOLOGY

Throughout the trip we heard that Chairman Mao stressed prevention and treatment in health care. In China, as elsewhere, prevention of cancer has drifted away from etiology toward detection. By identifying causes and avoiding them or blocking their effects, one can prevent disease; this concept is different from one's detecting the disease early when treatment is most effective.

In Shanghai and Peking, we heard that cancer has

TABLE 4.—*Frequencies of new cases of cancer by age group, Shanghai City Tumor Registry, both sexes combined, 1975, compared with those among U.S. whites, 1969–71 (27)*

0–14 yr			15–44 yr			45–64 yr			65+ yr		
Site or type	Frequency, %		Site	Frequency, %		Site	Frequency, %		Site	Frequency, %	
	China	U.S.		China	U.S.		China	U.S.		China	U.S.
Leukemia	29.65	33.47	Liver	10.83	0.47	Stomach	19.44	2.44	Lung	23.20	12.27
Nervous system [a]	20.00	16.45	Stomach	10.83	1.27	Lung	15.13	16.24	Stomach	22.25	4.50
Lymphoma	12.41	10.01	Breast	10.35	20.32	Liver	12.08	0.60	Esophagus	10.71	1.01
Bone	5.52	4.13	Thyroid gland	9.16	0.25	Cervix [b]	11.11	9.15	Liver	8.68	0.76
Soft tissue	4.83	5.00	Rectum	6.07	1.59	Esophagus	7.29	1.03	Cervix [b]	5.77	4.37
Other	27.59	30.94	Other	52.76	76.10	Other	34.95	70.54	Other	29.39	77.09

[a] Brain and nervous system.

[b] Probably includes uterus.

multifactorial interacting causes, which are unlikely to be identifiable. The Chinese argued that the decline in stomach cancer rates in the United States, for unknown reasons, was due to such interactions. This experience, they said, illustrates that one need not identify causes to achieve prevention.

In accord with this point of view, the Chinese are attempting multifactorial intervention to reduce the frequency of esophageal carcinoma in Linhsien County: the addition of molybdenum to the soil, reduction of nitrates in the water supply, avoidance of moldy food, and elimination of pickles in the diet. Hopefully, cancer-inducing interactions that cannot be demonstrated experimentally will thus be interrupted. The concept has merit when well founded, except that it tends to direct attention away from etiology, just when the Chinese could be on the verge of identifying the transportable carcinogen that induces gullet cancer in chickens and perhaps esophageal cancer in man.

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ADDENDUM

A Chinese Cancer Delegation visited the United States for 3 weeks beginning in mid-April 1978, under the sponsorship of the American Cancer Society. Our previous contact with members of the Delegation greatly enhanced the exchange of information. We learned that in March 1978 four areas in medicine were selected for rapid advancement during the next 20 years: oncology, cardiology, family planning, and acupuncture. Cancer epidemiology is one of the high priority items in oncology.

GUEST EDITORIAL—by J. C. Wagner

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JNCI 46:v–ix, 1971

Asbestos Cancers

TUMORS ASSOCIATED with exposure to asbestos dust have attracted considerable attention in recent years, largely because they have been of interest to many medical and scientific disciplines. The work illustrates the value of international collaborative studies in which research institutes, university departments, government services, and industry have all played a part.

There are several types of asbestos. All are fibrous silicates and have similar properties. They are used mainly to produce fireproof insulation or as a matrix for cement or plastics in building materials. Asbestos has hundreds of other uses and is essential in our industrial society. For example, the brakes of automobiles depend on the abrasive nature and heat resistance of asbestos. The inhalation of asbestos dusts can lead to pulmonary fibrosis and carcinoma of the lung or to the development of diffuse mesotheliomas of the pleura and peritoneum. The problem has been to discover which type or types of asbestos are responsible for the development of the tumors; if a specific variety could be incriminated, then its use could be discouraged and that of the less harmful varieties recommended.

Three types of asbestos are important commercially: chrysotile (white), most abundant and accounting for about 95% of the total; amosite (brown), and crocidolite (blue). A small amount of anthophyllite, a talclike fiber, is produced in Finland. Chrysotile is widely distributed in the main mining areas of Canada, Russia, Southern Africa, and Cyprus. Crocidolite is found in Cape Province and Transvaal in South Africa, and until recently was produced in Western Australia; a deposit is being developed in Bolivia. Amosite is mined only in the Transvaal in South Africa, but a large deposit of similar fiber has just been discovered in India.

The physical nature of the chrysotile fiber from different deposits varies considerably, as do the associated minerals found in the ore. However, after the fiber has been separated from the ore and processed in the mills, all chrysotile fibers have a soft, wavy appearance. In contrast, the crocidolite and amosite fibers are much more rigid, straight, and larger in diameter. They are also more resistant to acids and alkalis. From 1900, crocidolite was in demand in the shipbuilding industry because chrysotile was not a good insulating material when exposed to sea water. Crocidolite is now used mainly to form a matrix in chrysotile asbestos cement products, pressure piping, and automobile battery boxes. Amosite binds well with plastics and is used in the manufacture of floor

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Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors will welcome suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

tiles and in decorative insulations to replace timber in passenger ships. The use of asbestos has increased tremendously in the last 20 years and the annual consumption is over four million tons.

Asbestosis, the fibrosis of the lung caused by inhalation of asbestos, was first detected at the turn of the century in France and Britain, but it was not until the early 1930's that it was shown to be a major occupational hazard in the asbestos textile industry (*1*). The first suggestion that there might be an association between asbestosis and bronchial carcinoma came from pathologists in Britain and the United States in 1935. This was supported in Britain by the Inspector of Factories, who reported that in 1947 (*2*) 15% of all death certificates for males, on which asbestosis was mentioned, stated that death was due to carcinoma of the lung. This excessive risk was confirmed in 1955 by Doll (*3*) who showed that those first employed before 1930 in "scheduled" occupations in a textile factory had a death rate from lung cancer 10 times greater than that of the general population. Later studies on the same population confirmed that with improvement in factory conditions the incidence of carcinoma dropped considerably. This suggested that carcinoma of the lung was associated with the incidence of severe pulmonary fibrosis due to heavy asbestos exposure (*4*). In 1960, the situation was further complicated by our demonstration of the association between exposure to asbestos dust and the development of diffuse mesotheliomas of the pleura and peritoneum (*5*). This has several intriguing features: 1) These rare tumors were associated only with exposure to the crocidolite (blue) asbestos mined in the North West Cape Province, and not to the chryosotile and amosite mining areas of Southern Africa or to the crocidolite deposit adjoining the amosite area. 2) The association was with exposure to asbestos dust and not necessarily with the pulmonary fibrosis of asbestosis; a number of the cases were environmental rather than occupational exposures. 3) The average latent period between initial exposure to asbestos dust and development of the tumor was over 40 years. This was the first indication that the different types of asbestos varied in their biological effect.

Crocidolite has been used in Britain since 1900, but by 1962 the Inspectorate of Factories' records of 130 cancers of the lung in asbestos workers contained no cases of mesothelioma (*6*). A search was therefore made in the departments of pathology and surgery, and cases were referred to a panel of pathologists formed to establish criteria for the diagnoses of these previously rare tumors (*7–9*).

By 1964 it was clear that mesotheliomas had occurred and were increasing in number (*10*), but as most British asbestos workers had been exposed to more than one type of fiber, it was impossible to implicate a single type. More than 600 mesotheliomas are now recorded in the United Kingdom—the majority associated with exposure to asbestos (*11*).

To study the effect of exposure to one type of fiber, epidemiological studies were required in the mining areas. A comparison of the South African crocidolite mines with the chrysotile mines in Canada was needed. International coordination for this was possible through a UICC working group which, in 1964, reviewed the problem of asbestos and cancer and recommended lines for further work. A committee to implement these proposals was formed (*12*). In this way it was possible to initiate international epidemiological surveys, develop a new radiological classification (*13*), organize international and national pathology panels, and prepare, characterize, and distribute reference samples of asbestos dust for experimental work (*14*). It is hoped that the working party will reconvene in the fall of 1972 to consider how successfully the recommendations have been implemented and to report on the information that has been obtained concerning "Asbestos and Health."

Meanwhile, preliminary information is available on several aspects of the problem. A survey of Quebec asbestos miners has been undertaken by epidemiologists of McGill University with support of the Quebec Asbestos Mining Association. They conducted a remarkably complete follow-up of about 12,000 workers and showed a slight excess of lung cancer in the most heavily exposed group. Despite the heavy general air pollution by chrysotile dust over many years, the crude death rates for lung cancer in the population of the two mining towns are less than the death rate for the Province of Quebec (*15*). No mesotheliomas were recorded in the mining population. On the whole, the situation was entirely different from that in the asbestos fields in the North West Cape.

In the United States, prospective mortality studies, with use of social security records, showed an excess risk of bronchial cancer in those working in an asbestos-textile factory just before the Second World War (*16*). In New York insulation workers, death rates from lung cancer were also about 7 times those expected (*17*). Mesotheliomas of the peritoneum were observed among those on the union books in 1942, but more recently employees show an increased incidence of pleural tumors. Carcinoma of the lung was particularly prevalent among heavy cigarette smokers, and the effect appeared to be multiplicative (*18*), but there was no correlation between cigarette smoking and the development of the mesotheliomas. Information about the type of asbestos used in the past by these workers is incomplete, but crocidolite, if used at all, was present in very small amounts compared to chrysotile and amosite.

The Soviet Union is second only to Canada in chrysotile production. At a meeting in Dresden in 1968, Professor Kogan indicated an increased incidence of carcinoma of the lung in the asbestos area near Svedlovsk. Professor J. C. McDonald, who was in charge of the survey in Quebec, has recently discussed results with Kogan, and the situations in the Ural and Quebec asbestos fields are similar, with slight increase in carcinoma of the lung among heavily exposed workers, and with no mesotheliomas. Similar results have been obtained in Finland among the anthophyllite miners.

In contrast, mesotheliomas have been reported from shipbuilding ports, particularly in dockyards in Britain, the United States, Germany, and Holland. The whole situation was considered in Britain by a panel of experts under the chairmanship of the Chief Medical Inspector of Factories. Their report, "Problems Arising from the Use of Asbestos" (*19*), concluded there was sufficient evidence to suggest "other types of fibre should be substituted for crocidolite wherever possible." The law concerning the use of asbestos has been changed, and since 1970 the use of crocidolite has been restricted in Britain. It is disquieting to realize that, while the use of crocidolite has dropped rapidly during recent years in Britain, there has been a steady increase of the importation into the United States.

Possible reasons for the role of crocidolite in the production of mesotheliomas have been considered. In animal experiments, the tumors can be produced easily after intrapleural inoculation of various types of asbestos. There is no significant difference in the incidence of tumor production if chrysotile or crocidolite fiber is used (*20*).

The only clues have come from the physicists who have considered the aerodynamics of the different types of asbestos in relation to inhalation. To a first approximation, the rate of fall of an asbestos fiber depends on its diameter, not its length, so that when asbestos fibers are inhaled, deposition due to sedimentation is almost independent of length (*21*). This explains why long fibers (up to about 200 μ) can penetrate to the alveolar regions if they are thin enough (<3 μ diameter). On the other hand, deposition by interception or wall collision, which is important in narrow airways, depends almost entirely on fiber length. Chrysotile fibers, resembling stretched coils, are readily deposited by interception, especially at bifurcations, since the curvature increases the collision cross-section area. Amphibole fibers, which are straight, are less readily deposited by interception, since they tend to turn into the direction of the streamlines in the airflow and thus have small collision cross sections. In an experiment in which rats were exposed by inhalation to dust clouds generated from the UICC reference samples, the retention of amphiboles was 6 times greater than for chrysotile (*22*, *23*). A similar result was obtained when asbestos aerosols were sucked through hollow lung casts in which the airways were left open at the respiratory bronchioles (*24*). This ratio for the different types of fiber may be substantially higher in industry. Many chrysotile mining areas are characterized by dense clouds of dust, but a substantial proportion of particles are in the form of large flocks which settle quickly and are available for inhalation only during a short period. Even in chrysotile mills, enough fiber may reach the bronchus to cause asbestosis and carcinoma of the lungs, but, in general, exposure to the amphiboles is more likely to produce these diseases. The human risk of developing mesothelioma of the pleura from chrysotile may well be due to its curly nature which permits most of it to reach the pleura. Quantitative studies of the distribution of different types of fiber within the lungs after inhalation need further development.

By using the electron microscope for measurement, physicists have shown that the fibers of crocidolite mined in the North West Cape and Australia are much thinner and shorter than those of the crocidolite and amosite mined in the Transvaal and consequently have a much greater chance of reaching the pleura. This hypothesis could account for the high rate of pleural mesotheliomas in the North West Cape and their presence in Australia where mining began in 1945, compared to only one case in the Transvaal.

Carcinomas of the lung are associated with the moderate-to-severe fibrosis of asbestosis, but there is no evidence as yet to implicate a particular type of asbestos or to measure the relative risks for exposure to one type of fiber. It would seem that excessive exposure to all types of fiber could be responsible. There is evidence to support the contention that the incidence of carcinoma can be greatly reduced by good plant hygiene. Mesotheliomas appear to occur after slight exposures, particularly to crocidolite; the use of this type of fiber should therefore be abandoned if not essential. The position of amosite is not yet clear, so that a high standard of dust control is required when it is used. The use of chrysotile may carry only a small risk when the dust control is at a level which is now practical and economic. However, there is a considerable difference between the chrysotile from various deposits, and preliminary results of current intrapleural experiments (*25*) indicate that chrysotile from certain individual mines are more reactive than others. Thus the need is still urgent for further studies on the biological effects of the various types of asbestos to find a relatively inert fiber for industry. Further work on the effect of crocidolite on the mesothelial cells may provide useful information in the study of carcinogenesis.

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ADDENDUM

In the period since this Editorial was published, the main interest in the cancers associated with exposure to asbestos dust has remained the mesotheliomas of the pleura and peritoneum and the carcinomas of the lung. A slight increase in carcinomas of the gastrointestinal tract has been confirmed in two studies, and an increase in carcinoma of the larynx has been suggested but not confirmed in the major investigations in Britain and the United States. A possible association with carcinoma of the ovary remains unsolved due to the problem of separating the peritoneal mesotheliomas from the ovarian tumors.

The two major problems concerning the mesotheliomas remain: Are the mesotheliomas associated specifically with exposure to crocidolite asbestos dust? Is the general public at risk in the development of these tumors? The available evidence for these controversial subjects was discussed in detail at the International Agency for Research on Cancer (IARC) Conference on "Biological Effects of Asbestos" held in Lyon in 1972 (*1*) and has been reconsidered on numerous occasions as the results of more detailed investigations become available (*2*). It is generally accepted that exposure to crocidolite dust can result in mesothelioma, and the consensus among authorities (based on available evidence) is that other types of fiber are also implicated, though to a lesser degree. The possible risk to the general population is a problem of considerable concern. All the information available at the time was considered at the 1972 IARC Conference, and it was the opinion of the Advisory Committee to the Director of the IARC that no risk to the general public was evidenced (*3*). Although this opinion has been challenged over the intervening years, no authentic case of this nature has been produced. Mesotheliomas have occurred in some paraoccupational groups, e.g., persons living in the vicinity of the crocidolite mines and mills, in the immediate vicinity of factories in industrial countries, and those having household contacts.

Carcinoma of the lung in asbestos workers, as previously stated, occurs after excessive exposure to all commercially produced types of asbestos. In recent years, evidence of a dust–dose relationship has been established, and the multiplicative effect of cigarette smoking has been confirmed.

Ingestion as a cause of nonoccupational risk has been considered. Three sources cause concern: the presence of chrysotile fibers in beverages and pharmaceutical preparations following filtration through asbestos pads; fibers in drinking water, which has passed through asbestos-lined cement pipes; and fibers from water passing through, or being obtained from, sources which contain asbestos or associated mineral fibers. Intensive investigation of these possible sources has not revealed any evidence of an associated increase in peritoneal mesotheliomas or cancers of the gastrointestinal tract.

A broadly based review of the whole field of investigations into the problem of the asbestos cancers will be found in the recent IARC Monograph on Asbestos (*4*).

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EDITORIAL

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Cancer Research by the Atomic Bomb Casualty Commission

MUCH OF what is known about radiation-induced cancer in man comes from the work of the Atomic Bomb Casualty Commission (ABCC), a collaborative U.S.-Japanese research organization in Hiroshima and Nagasaki. To an impressive series of publications on cancer over the past two decades, ABCC recently added a trio of papers of considerable interest. They concern the minimum leukemogenic dose among survivors (*1*), intrauterine exposures in relation to childhood cancer deaths (*2*), and a recent excess of deaths from cancer other than leukemia among persons heavily exposed during childhood (*3*).

Leukemia

Previous studies of atomic-bomb survivors revealed an excess of leukemia (*4–6*). A dose-response relationship was demonstrated; a peak in occurrence was observed in 1951, 6 years after exposure; the type of leukemia induced was acute lymphocytic, acute myelogenous or chronic myelogenous, but notably not chronic lymphocytic; and the effect was greatest in those exposed during childhood (*5, 6*).

In the most recent analysis of leukemia data at ABCC (*1*), the lowest dose category with an increased frequency was 20–49 rad, considerably less than the previous low of about 80 rad (*6*). The effect at 20–49 rad was found only in Hiroshima, where neutrons constituted a substantial fraction of the total dose. In Nagasaki, exposure to neutrons was very small, and no cases of leukemia occurred among survivors exposed to 5–99 rad (*1*). Leukemogenesis at lower doses in Hiroshima may thus be due to the greater relative biological effectiveness (RBE) of neutrons, as compared with γ-radiation. Other differences between the cities (*e.g.*, genetic) may be involved, however.

The observations by ABCC have demonstrated that leukemia can cluster in time and space as a result of an environmental agent. Furthermore, there was a concomitant cluster of a congenital malformation (small head circumference with mental retardation) as a result of the same agent (*7, 8*). Exposure to ionizing radiation is obviously both leukemogenic and teratogenic. The capacity of irradiation to produce point mutations, well demonstrated in various other species, was not of sufficient magnitude to be demonstrable in the atomic-bomb survivors (*9–12*). Somatic chromosomal damage, however, has been shown to persist until now among atomic-bomb survivors; such damage was found even after exposure during the first trimester of intrauterine development, before the lymphocytes can be recognized as such (*13–17*).

Intrauterine or Preconception Exposures

Jablon and Kato (*2*) recently made a major finding at ABCC when they tested the estimate of Stewart and Kneale (*18*) that if 1 million children were each exposed to 1 rad *in utero*, there would be 300–800 extra cancer deaths before 10 years of age. The cumulative maternal dose in Hiroshima and Nagasaki was 34,933 person-rad. If the fetal dose was half this amount, and it was probably more, Stewart and Kneale's estimate would yield

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an average of 18.4 extra cancer deaths, with a lower limit of 5.2, as compared with only 0.25 excess observed among the ABCC *in utero* group. Thus the data from Japan do not support the Stewart and Kneale estimate, which was based on the number of obstetric X-ray films taken.

There are several possible explanations for these dissimilar results, but the lack of agreement raises further questions as to the capacity of very low doses of intrauterine X ray to induce all forms of childhood cancer by about 50% (*19*, *20*). Interpretation of the results seems further clouded by a report from an expert group of U.S. epidemiologists (*21*), who found in a collaborative study conducted in Baltimore, Minneapolis, and New York State, excluding New York City, that a similar (60%) excess of leukemia occurred in children whose mothers reported diagnostic X-ray exposures as long as 10 years before conception of the child. Half as great an increase (30%) was also observed with respect to paternal diagnostic irradiation before conception of the child. One must conclude that the leukemogenic effect, if real, was genetic—even though a comprehensive study involving six indicators of genetic damage in the F_1 generation (conceived after the explosions) in Hiroshima and Nagasaki showed no clear influence of radiation exposure (*9–12*). Also, no excess of leukemia was found among these children (*22*) whose parents were exposed to a range of dose much greater than that in diagnostic radiology.

Other Cancer

In studies of other cancers by ABCC, thyroid carcinoma had increased among persons who received large doses of radiation (*23*), whereas cancer of the lung (*24*) and breast (*25*) occurred in relatively small excesses of uncertain relationship to radiation exposure. No other site-specific cancers have yet been shown by ABCC to be radiation-induced. Study of death certificates for survivors of all ages suggested an excessive mortality from all cancer other than leukemia through 1962–66, but not through 1954–62 (*26*). The results of an extension of this study through 1970, soon to be published, will be of great interest.

In the most recent publication on cancer from ABCC, Jablon *et al.* (*3*) reported excessive malignant neoplasms, other than leukemia, in the last few years among persons *under 10 years of age* at the time of the bomb and whose dosage was 100 rad or more, or unknown. The results were similar in a death-certificate series in which 8 cases were observed as compared with 1.1 expected, and in a clinically diagnosed series in which 6 cases were observed as compared with 1.3 expected. The ages at the time of diagnosis or death were 16–31 years. This development may represent the beginning of a larger excess soon to become apparent.

Other Features

ABCC has biomedical significance beyond its contributions concerning radiation effects. It is a leading producer of epidemiologists and has familiarized many other medical scientists with the research value of the epidemiological approach. It is a prospective population-based study with standard observations on tens of thousands of people for over two decades—useful not only for studies of radiogenic cancer but of other cancers and disease in general. It affords opportunities for linking various health records on the same individuals. Several unusual features of life in Japan have been effectively used by ABCC to enhance scientific research. Among these are the family registry system (*koseki*), which permits easier follow-up than usual (*27*, *28*), and a high rate of cousin marriage (about 7%), which permits study of specific diseases in relation to parental consanguinity—a measure of the influence on health of rare recessive genes (*28*). In all these functions, ABCC has been a long-term cooperative international endeavor. The creation of resources to achieve these aims has repeatedly been urged. At ABCC they already exist.

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ADDENDUM

In the 7 years since the Editorial appeared, the ABCC has been superseded by the Radiation Effects Research Foundation (RERF), under which Japanese leadership has been greatly strengthened and funds come equally from both nations. Surveillance of cancer occurrence and pathology has been aided since 1972 by the establishment of Tumor Tissue Registries by the Prefectural Medical Associations in Hiroshima and Nagasaki, with funds provided by the National Cancer Institute of the United States. Continuation of the studies by ABCC/RERF has revealed new findings on carcinogenesis in particular.

The evidence that radiation contributes to cancer of the lung and breast among survivors has substantially increased (*1–3*). The data on breast cancer show a linear dose–response relationship consistent with an effect even below 10 rads. The carcinogenic response is age-related, the relative risk being greatest among females exposed as teen-agers. Various histologic types were observed (*3*).

A series of analytical studies of mortality among atomic bomb survivors had been extended for an additional 4 years, 1971–74 (*4*). The results show that the only cause of reduced life-span has been cancer and that this effect is not a manifestation of accelerated aging. The study also showed disappearance of the ominous signs of an epidemic of cancer among young adults who had been exposed to the bomb under 10 years of age.

A review of all findings from studies of the atomic-bomb survivors in the 30 years since their exposure has been published (*5*). The report concerns dosimetry, acute and late biologic effects (genetic or somatic), and future research plans. The observations made in Hiroshima and Nagasaki provided a main contribution in the evaluation of low-dose radiation effects by the United Nations Scientific Committee on the Effects of Atomic Radiation, published in 1977 (*6*), and by the Advisory Committee on the Biologic Effects of Ionizing Radiations (National Academy of Sciences–National Research Council), to be published in early 1979.

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EDITORIAL—by Robert W. Miller, M.D.

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Radiation-Induced Cancer

THERE IS NO DOUBT that ionizing radiation can induce cancer in man. The effects differ according to the type of neoplasm, age of the subject, and dose of radiation.

Leukemia

Soon after the discovery of X-rays, case reports of leukemia in radiation workers appeared (*1*). Confirmation promptly came from animal experimentation (*2*). Review of death notices published weekly in the Journal of the American Medical Association revealed that the frequency of leukemia among physicians was 1.7 times that of the U.S. white male population (*3*). A refinement of this study was reported independently by Ulrich (*4*) and March (*5*). They studied the death notices for radiologists as compared with other physicians and found a ninefold greater frequency of leukemia among radiologists. In their comparison, they did not correct for age. In the time interval covered in the studies, radiology was a new specialty, and radiologists as a group were young, usually less than 45 years old, whereas other physicians were much older. Hence the relative frequency of leukemia in the young group was substantially greater than that of the older group. When correction was made for age, the ninefold excess among radiologists diminished to threefold (*6*). The effects were still present but substantially less than the original estimates.

Total-body exposures.—After the explosions of the atomic bombs in Hiroshima and Nagasaki, a cohort study of the survivors was initiated by the Atomic Bomb Casualty Commission (ABCC). Thus important details of radiation-induced leukemia could be defined through a prospective study. In 1951, 6 years after the explosions, a peak in the frequency of leukemia was reached, more pronounced for the acute leukemias than for chronic myelogenous leukemia (CML) (*7*). The frequency of CML fell to about normal rates 15 years after exposure, whereas acute leukemia continues to occur excessively, though the rates have declined markedly since the peak in 1951.

Notably in Hiroshima no cases of chronic lymphocytic leukemia (CLL) were observed. This form of leukemia has a low frequency among Japanese, as contrasted with people in Western countries, among whom it constitutes about 30% of all adult leukemia (*8*). However, among British men given radiotherapy for ankylosing spondylitis, leukemia has markedly increased, but again not CLL (*9*). Thus radiation induces several forms of leukemia in man, but not the chronic lymphocytic form of the neoplasm.

Acute lymphocytic leukemia was radiation induced in survivors of Hiroshima and Nagasaki, only if they were under 20 years old at the time of the bomb (*10*). Susceptibility to leukemia generally

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Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

was about 3 times greater when exposure occurred under 10 years of age as compared with 10–19 years (*11*). However, CML was particularly frequent in the older age groups (*10*). One must conclude that host factors related to age determine the cell type of leukemia induced by radiation.

Partial-body exposures.—Partial-body radiation is also leukemogenic, as indicated by the study of men treated with X-ray for ankylosing spondylitis (*9*). They exhibited a tenfold excess of the neoplasm, with a peak 6 years after exposure and a dose-response effect—duplicating the epidemiologic features of leukemia among atomic bomb survivors. After radiotherapy for enlarged thymus in infancy, 6 children developed leukemia as compared with 2.02 expected by chance (*12*). Also, following X-ray epilation as treatment for tinea capitis in childhood, 4 cases of leukemia were observed among 2,404 in the treated group as compared with none among 1,540 controls (*13*).

Lowest leukemogenic dose.—The best information on the minimal leukemogenic dose in man comes from study of the atomic bomb survivors (*14*). The lowest dose category at which an increase in the neoplasm could be detected was 20–49 rads. The effect was noted in Hiroshima, where the neutron dose was substantial, but not in Nagasaki, where it was small. The dose-response effect for leukemia in each city can be made similar if the relative biological effectiveness (RBE) of neutrons is set equal to 5. The lowest dose category in Hiroshima at which an increase in leukemia can be detected then becomes 32–85 rem (RBE×rad at various dose levels) (*15*). The difference in leukemia occurrence in the 2 cities, however, may not be due to neutrons, since other differences in the populations exist, e.g., genetic differences. Consequently one can say that the lowest leukemogenic dose yet known is somewhere between 20–49 rem (RBE = 1) and 32–85 rem (RBE = 5).

In Utero and Preconception Induction of Cancer

In 1956 Stewart and associates (*16*) published a preliminary report describing a twofold excess of leukemia and other cancer among children whose mothers received diagnostic X-rays, during the relevant pregnancy. A definitive report published 2 years later (*17*) showed that the risk of cancer among the irradiated group was about doubled for 6 of 8 categories of childhood neoplasia, except for myeloblastic leukemia and lymphoma.

The history of irradiation was obtained by interview from mothers of *a*) 619 children who died of leukemia, *b*) 680 who died of other cancer, and *c*) an equal number of controls (children without cancer) matched by age, sex, and locality. All deaths in the case group occurred before 10 years of age, 1953–55.

In 1962 MacMahon (*18*) reported similar results from a study with objective evidence of maternal radiation exposure rather than reliance on interviews, which may be unavoidably biased. Through the use of obstetric records in 37 large maternity hospitals in New England, 1947–54, he determined the frequency of diagnostic X-ray study of the mothers' abdomens during pregnancy for 569 children who subsequently died of cancer, as compared with a 1% systematic sample of all other births in the same hospitals. The results indicated that after such exposure the relative risk of childhood leukemia was increased by 40%, cancer of the central nervous system by 60%, and all other cancer by 50%. No relation was found between neoplasia and recorded complications of pregnancy. The oncogenic effect of X-ray was apparently exhausted by 8 years of age, in contrast to its persistence through the entire span (up to 10 years of age) in the study of Stewart et al. In comparing his results with all others available to date, MacMahon noted that those showing no relation to X-ray exposure [including a prospective study by Court Brown et al. (*19*)] were unlikely, because of the relatively small sample sizes, to reveal an increase in relative risk of only 40–60%. Subsequently, a prospective pilot study by Diamond and Lilienfeld (*20*), involving follow-up of about 20,000 children exposed to diagnostic radiation in utero and 40,000 controls, revealed among whites ". . . a nearly two-fold increased risk of dying (from all causes) during their first ten years of life." No excess occurred among the black children, who made up about half the sample studied. Leukemia caused the deaths of 6 white children as compared with 2 expected; no such deaths were among in utero-exposed black children. Neither ethnic group experienced an excess of other cancers.

Equal induction of each form of childhood cancer.—In 1968 Stewart and Kneale (*21*) published a further report on their data, which showed that each of 6 categories of childhood neoplasia exhibited an equal increase in relative risk after maternal abdominal exposure to X-ray during pregnancy (table 2 of their paper). The classes of neoplasms involved (leukemia, lymphoma, neuroblastoma, cerebral tumors, Wilms' tumor and all other cancer) differ markedly from one another epidemiologically (*22*), and the biologic plausibility that low-dose intrauterine irradiation would increase the frequency of each by about 50% was questioned by Miller (*23*).

Establishment of a causal relationship would have been aided by demonstration of a specificity of effect (for one rather than all forms of childhood cancer), a dose-response effect, and a consistency with data from animal or other laboratory experimentation. Interpretation of the results seemed further clouded by a previous report by a group of U.S. epidemiologists (*24*) who found, in a collaborative (tri-State) study conducted in Baltimore, Minneapolis, and New York State excluding New York City, that a similar (60%) excess of leukemia occurred in children whose mothers reported diagnostic X-ray exposures up to 10 or more years before conception of the children. Half as great an increase (30%), of borderline significance, was also observed with respect to paternal diagnostic irradiation before conception of the child. One must conclude that the effect, if real, was heritable. It seems unlikely due to a genetic influence, since a comprehensive study involving 6 indicators of genetic damage in the F_1 generation in Hiroshima and Nagasaki showed no detectable influence of radiation exposure (*25–27*). Also, no excess of leukemia was observed in the F_1 generation (*28*) though the radiation dose range was much greater than in the diagnostic X-ray studies. In addition, cytogenetic studies of atomic bomb survivors in Japan showed complex chromosomal abnormalities if exposure occurred in utero or in later life, but not in the F_1 generation (conceived after the explosions) (*29–32*). Thus the claim that preconception radiation is leukemogenic could not be linked to the genetic mechanism, chromosomal abnormality, that characterizes persons known to be at high risk of leukemia (*33*).

The authors of the tri-State study, further examining their data on preconception exposures, concluded that other factors interacted with radiation to increase the risk of leukemia among children from 1–4 years old (*34*). Thus, for example, the relative risk rose about fourfold when such irradiation occurred in conjunction with a maternal history of reproductive wastage *and* the child had at least one viral infection more than 12 months before the onset of leukemia. The numbers of cases involved in this particular estimate, however, were small. Statistical significance at the 5% level was attained when 2 radiologic factors (preconception *and* intrauterine exposures) were related to 1 or 2 pathologic factors (childhood virus infection or maternal reproductive wastage), or when 1 radiologic factor was related to both pathologic factors. Surely, as the investigators said, these observations require confirmation (which will not come easily because of the massive effort required to collect such data).

As stated earlier, finding that preconception irradiation is associated with an excess of childhood leukemia complicates interpretation of cancer occurrence after small intrauterine doses of X-ray. It is difficult to rationalize such similar results, whether exposure was before conception, as found in one well-executed study, or intrauterine, and if the latter, whether the event observed was leukemia or any other childhood cancer.

Linear dose-response relationship.—Stewart and Kneale (*35*) subsequently described a linear relationship between radiation dose (0–5+ abdominal films) and the excess in cancer risk under 10 years of age. (Through the use of a different method Newcombe and McGregor (*36*) confirmed the linear relationship.) Stewart and Kneale estimated that ". . . among 1 million children exposed shortly before birth to 1 rad of ionizing radiations there would be an extra 300–800 deaths before 10 years of age due to radiation-induced cancer (mean 572 deaths, standard error 133)." This estimated number of extra cancer deaths per million rad of intrauterine exposure could be tested in another situation: the survivors of Hiroshima and Nagasaki. The study, recently reported by Jablon and Kato (*37*), concerned 1,250 children exposed in utero to less than 500 rads. The accumulated dose was 34,933 person-rads. Under the conservative assumption that half the dose was attenuated by the mothers' bodies, 18.4 extra cancer deaths of children under 10 years of age would have been expected, according to the Stewart and Kneale estimate (lower limit=5.2) as compared with essentially no extra cancer deaths observed among children exposed in utero to the atomic bombs. To explain the lack of agreement between the 2 studies, Jablon and Kato suggested that Stewart and Kneale may have overestimated the cancer induction rate; that the dose-response curve may be linear at low doses and concave at higher ones, as might occur if abortions were induced by radiation, i.e., a competing risk; that high-energy atomic radiation may be a less effective carcinogen than is low-energy X-ray as used for diagnosis; or that factors other than X-ray distinguish the irradiated from the nonirradiated fetus; i.e., low-dose X-ray exposure is not the cause of the childhood cancer, and the diagnostic procedure merely indicates that the pregnancy differed from normal.

As to intrauterine exposures, until a causal relationship to an increase in childhood cancer can be definitively excluded, medical and occupational irradiation of pregnant or potentially pregnant women should be limited insofar as possible.

Susceptibility to Cancer After Childhood Irradiation

As already mentioned, leukemia was about 3 times more frequent among children exposed to the atomic bomb before 10 years of age, as compared with those who were 10–19 years old (*11*). Recently, other cancers have occurred excessively among the younger group when the dose was 100 rads or more, or unknown (*38*). The results were similar in a death certificate series in which 8 cases were observed as compared with 1.1 expected, and in a clinically diagnosed series in which 6 cases were observed as compared with 1.3 expected. The ages at diagnosis or at death were 16–31 years. This development may represent the beginning of a larger excess to be revealed in the next few years.

Other Radiogenic Cancer

An increased mortality from cancer has been observed among heavily exposed atomic bomb survivors from 1950–70, except for 1955–59 (*39*). There was no specificity as to cell type.

Similar findings were reported earlier from the study of British men treated with X-ray for ankylosing spondylitis (*9*). There was about a twofold increase in the frequencies of cancers within the field of radiation, but not outside it.

Thyroid cancer.—Leukemia is the neoplasm most readily induced in man by ionizing radiation. The second most readily induced is thyroid cancer. A retrospective study in 1950 revealed that 10 of 28 persons with thyroid cancer had had thymic irradiation in infancy (*40*). A prospective study reported in 1955 confirmed the finding (*41*), and data collected in later years revealed that among 2,878 persons in the X-ray-treated group, 19 had developed thyroid cancer as compared with 0.1 expected; benign tumors of the thyroid were found in 22 as compared with 1.6 expected (*12*).

In the Japanese survivors of the atomic bombs, an excess of thyroid cancer was found in relation to dose, and more frequently among women than men (*42*). Among 53 Marshallese exposed to fallout doses of 175 rads, 19 developed benign adenomatous nodules and 3 females developed malignant tumors (*43*). Special susceptibility to benign tumors was apparent early in life, since, among 67 persons exposed under 10 years of age to a much smaller dose, 69–74 rads, 4 developed benign tumors as compared with none among 46 persons over 10 years of age when exposed. The thyroid tumors among the Marshallese were primarily due, of course, to radioactive iodine in the fallout.

Lung cancer.—Inhalation of radioactive dust can undoubtedly induce lung cancer in man, the most notable evidence being from the study of uranium miners (*44*). A relation between the neoplasm and *external* radiation is far less convincing. Among the Japanese survivors of the atomic bombs the excess was about twofold (*45*), but even with subsequent data the number of lung cancers was sparse. There was no clear dose-response effect, and interpretation was complicated by inconsistencies in the data and lack of information about cigarette smoking (*11*). A similar excess, however, has also been found in the British study of men who received therapeutic irradiation of the spine for ankylosing spondylitis (*9*). A study of U.S. radiology technicians in the military during World War II revealed that 17 had died of lung cancer as compared with 12 expected at national mortality rates (*46*).

Breast cancer.—The evidence of a relation between irradiation and breast cancer is also equivocal. After a clinical examination by ABCC, which revealed no neoplasia, 9 women developed breast cancer as compared with 3.7 expected (*47*). In a study of women who received pneumothorax for tuberculosis with evaluation by multiple fluoroscopies, 13 of 271 developed breast cancer, a statistically significant excess over normal expectation (*48*). Thousands of roentgens (R) were apparently involved. In another study, follow-up was made of 606 women treated with X-ray (75–1000+ R) for acute postpartum mastitis. Breast cancer developed in 13 as compared with 5.86 expected at rates estimated from the State tumor registry (*49*). The extent to which mastitis may be associated with breast cancer could not be evaluated. Thus an etiologic role of radiation was not established. Taken together, the 3 studies are insufficient to demonstrate that radiation has induced breast cancer in women.

Bone cancer.—Study of radium-dial painters has conclusively shown that radioisotopes can cause human osteosarcoma (*50*). Heavy external radiation (3000+ rads) has also induced this cancer (*51*). Lower doses have been associated with a benign neoplasm, osteochondroma, after doses of about 500 rads for thymic enlargement in infancy (*12*).

Miscellaneous cancers.—High therapeutic doses of radiation—thousands of rads—have induced fibrosarcoma and cancer of the skin, as in the treatment of retinoblastoma (*52*). Cholangiomas and hemangioendotheliomas of the liver were observed excessively after the use of Thorotrast in diagnostic radiology (*53*). Brain tumors were reported in 3 of 2,043 persons who underwent X-ray epilation for tinea capitis (*13*). The estimated dose to the brain was 50–150 rads. There is no supporting evidence from other studies of man to suggest that radiation induced these brain tumors.

Perspective

It has been relatively easy to demonstrate epidemiologically that various (but not all) forms of cancer can be induced by ionizing radiation in man. The same methods have implicated some chemicals (*54*) but as yet no viruses in the etiology of human cancer.

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ADDENDUM

Data on carcinogenesis due to ionizing radiation from the atomic bombs in Japan from sources other than the above Editorial have been reviewed by the United Nations Scientific Committee on the Effects of Atomic Radiation (*1*), by the Advisory Committee on the Biologic Effects of Ionizing Radiations, National Academy of Sciences–National Research Council, to be published in 1979, and in the proceedings of an international symposium (*2*).

Radiation induction of breast cancer has now been well documented not only among survivors of the atomic bombs (*3*) but also among patients treated with X-rays for acute postpartum mastitis (*4*) or given repeated fluoroscopic examinations of the chest for tuberculosis (*5*).

Radiotherapy for tinea capitis in children has induced thyroid carcinoma at an estimated dose of less than 9 rads (*6–8*) and brain tumors at about 140 rads (*6*). An epidemic of thyroid cancer has been found among middle-aged adults in the United States who received radiotherapy to the thymus as infants or to the head and neck for benign diseases in childhood (*9, 10*).

Iodine-131 in fallout from a weapons test in the Marshall Islands has caused benign or malignant thyroid tumors (*11*). Among 19 children exposed under 10 years of age, 2 infants had their thyroids ablated by iodine-131, and 15 others developed thyroid tumors, all but 1 of which were benign. Among 4 children exposed in utero, 1 developed a thyroid nodule.

Radium-224, given intravenously as empiric therapy for tuberculosis of the bone in the Federal Republic Germany about 30 years ago and the subject of a 1974 international symposium (*12*), has induced dozens of cases of osteosarcoma and benign bone tumors (*13*).

Hypersensitivity manifested as cellular lethality from ionizing radiation due to defective repair of DNA has been identified in persons with ataxia-telangiectasia. Patients with this genetic disorder have a predisposition to lymphoma and, when treated with conventional doses of radiotherapy, may develop severe acute reactions (*14*). Some evidence suggests that radiogenic tumors occur more often and earlier than usual after radiotherapy of patients with the nevoid basal cell carcinoma syndrome and the hereditary form of retinoblastoma (*15*).

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GUEST EDITORIAL by Robert W. Miller, *M.D.*[1]

JNCI 58:471–474, 1977

Relationship Between Human Teratogens and Carcinogens[2]

By the study of congenital malformations among patients with specific forms of neoplasia, a new understanding of the fundamental biology of cancer has been derived. The first clues generally come from observations made at the bedside by astute clinicians and involve disorders for which no animal models are known. In these instances, the malformations are genetically induced or idiopathic. No corresponding increment in understanding has yet come from the capacity of some *environmental* teratogens to induce cancer in man.

FROM CLINICAL OBSERVATIONS TO MOLECULAR BIOLOGY

AT is a genetically transmitted disease with a marked predisposition to lymphoma (*1*). In 1967, a child with AT and lymphoma was reported to have developed acute severe toxicity to conventional radiotherapy for the neoplasm (*2*). A second case was described the following year (*3*). No further cases were reported until 1975 (*4*), when a similar case was seen in Birmingham, England. There, the clinical observation could be put to certain laboratory tests. It was soon found that fibroblasts from patients with AT had much shorter survival times than usual after irradiation in culture (*5*), and that radiation damage to DNA was not properly repaired (*6, 7*). The defect in DNA repair has been attributed to a deficiency of γ-endonuclease.

In another genetic disorder (XP), there is defective DNA repair after damage produced by UV light due to a deficiency of UV-endonuclease (*8*). The enzyme deficiencies in AT and XP are nonoverlapping. UV damage to DNA is readily repaired in patients with AT; γ-ray damage to DNA is readily repaired in patients with XP. Both genetic disorders predispose to cancer—AT to lymphoma in particular (*1*), and XP to skin cancer (*8*).

Another bedside clue to oncogenesis concerns the excessive occurrence of bilateral (i.e., hereditary) retinoblastoma and second primary tumors, especially osteosarcoma. These second primary tumors are not due to therapy for the original cancer (*9, 10*). According to Knudson's hypothesis (*11*), in the heritable form of retinoblastoma all target cells in the retina, as well as certain other cells in the body, have sustained a prezygotic first step in a two-step process that leads to cancer. The second step occurs postzygotically (*11–13*). In the nonheritable form of retinoblastoma, both steps are thought to occur postzygotically in a particular cell by rare chance.

Children with the heritable type of retinoblastoma seem to have an increased susceptibility to radiotherapy-induced second primary cancers of the orbit after a shorter latent period than usual [i.e., as compared with radiogenic tumors that occur in children treated for various nonheritable neoplasms (*14*)]. In consequence of these observations, in vitro studies of fibroblasts from a patient with bilateral retinoblastoma and multicentric osteosarcoma are in progress to determine if there is a defect in DNA repair to account for the heightened susceptibility to second primary cancers which is increased after radiotherapy.

A FEATURE IN COMMON IN PERSONS AT HIGH RISK OF SPECIFIC CANCERS

Another variation on the same theme concerns children with certain congenital malformations that put them at high risk of a specific cancer. When a feature in common among these malformations has been sought, it has been found that congenital cytogenetic abnormalities characterize persons at high risk of leukemia [e.g., Down's, Bloom's, or Fanconi's syndromes (*15, 16*)], congenital cell-mediated immunodeficiency characterizes persons at high risk of lymphoma (*1*), and urinary tract anomalies are the feature in common among the variety of congenital disorders that predispose to nephroblastoma (*17*).

Knudson and Strong (*18*) found that the same rationale applies to nephroblastoma as to retinoblastoma—the heritable form is usually multifocal whereas the sporadic form is unifocal. Recently, Bove and McAdams (*19*) described aberrant metanephric differentiation as a concomitant finding in multifocal nephroblastoma and suggested that these lesions are genetically determined precursors of the cancer, i.e., the first (prezygotic) step according to Knudson's hypothesis (*11*). The second (postzygotic) step produces malignant transformation.

From these rarities, generalizations about the origins of specific cancers can be derived. Thus environmental causes of leukemia (ionizing radiation and benzene) are associated with long-persisting chromosomal aberra-

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Abbreviations used: AT = ataxia telangiectasia; XP = xeroderma pigmentosum; PCB's = polychlorinated biphenyls.

[1] Clinical Epidemiology Branch, National Cancer Institute, National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare, Bethesda, Md. 20014.

[2] Based in part on information developed at a Conference on Birth Defects: Detection and Prevention, held in Fort de France, Martinique, January 26–28, 1976, sponsored by the Institut de la Vie and the National Cancer Institute, National Institutes of Health.

Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

tions (*20*). Leukemia in the population at large is increasingly being linked with specific cytogenetic aberrations that escaped detection before the newer banding techniques were developed (*21*).

ENVIRONMENTAL CAUSES OF CANCERS AND MALFORMATIONS

Teratogens Not Known to be Carcinogenic in Man

From the foregoing, there appears to be a close link between human cancer and congenital malformations, but there is no such link when the causes are environmental rather than genetic or idiopathic. Table 1 shows the nine chemicals and five viruses that are teratogens but not as yet known to be carcinogenic in man. Only one of the viruses is thought to be carcinogenic (herpesvirus 2) (*22*), but several of the chemicals may well be. PCB's are particularly suspect. They are teratogenic in man (*25*) and produce hepatocellular carcinoma in rats (*26*). Not only do PCB's cross the placenta, but they concentrate in the fat of human milk (*27*). The long-term effects in man, if any, are unknown (*28*).

A useful resource for the study of long-persisting pollutants in Japan are dried umbilical cords, traditionally kept without preservatives for decades. Through chemical analysis of umbilical cords stored since 1927 through 1966 in the Minamata area, high levels of methylmercury could be demonstrated not only at the time of the known epidemic of neurological disorder in the mid-1950's, but also 20 years before that, when a similar episode had gone unnoticed (*29*). Possibly the preservation of dried umbilical cords in the United States, an inexpensive simple procedure, would permit evaluation in the future of cancer or other diseases (some of them previously unknown) in relation to early exposure to PCB's or other pollutants (*30*).

Carcinogens Not Known to be Teratogenic in Man

Table 2 shows the 31 chemicals, two parasites, and two forms of radiation for which there is some (at times scanty) evidence of human carcinogenicity. Cigarette smoking is highly carcinogenic and reduces the birth weight of the infant, but has not been shown to produce congenital anomalies (*31*). Such an effect might be detectable by careful examination of infants whose mothers smoked heavily during pregnancy. In this way the subtle early signs of teratogenesis from alcohol and from diphenylhydantoin were discovered (*see* below) after they had escaped detection by review of routinely recorded diagnoses. Other agents listed in the table may also prove to be teratogenic in man should circumstances make an evaluation possible.

TABLE 1.—*Agents known to be teratogenic but not carcinogenic in man*

Chemicals (*23*)	Viruses (*24*)
Aminopterin	Rubella
Antithyroid drugs	Cytomegalovirus
Busulfan	*Herpesvirus hominis* 1 and 2
Mercury, organic	Varicella
Methotrexate	Venezuelan equine encephalitis
PCB's	
Tetracycline	
Thalidomide	
Warfarin	

TABLE 2.—*Human carcinogens*

Agent	Ames test[a]	Site of cancer	Reference
Alcohol		Mouth, larynx, esophagus	(*32*)
Androgens		Liver	(*33*)
Aromatic amines		Urinary bladder	(*34*)
Auramine[b]	0		
Benzidine	+		
α-Naphthylamine	+		
β-Naphthylamine	+		
Magenta[b]			
4-Aminobiphenyl	+		
4-Nitrobiphenyl	+		
Arsenic, inorganic		Lung	(*34*)
Asbestos		Lung, mesothelium	(*34*)
Benzene		Bone marrow (leukemia)	(*34*)
Bis(chloromethyl) ether	+	Lung	(*34*)
Chloramphenicol	?	Bone marrow (leukemia)	(*35*)
Chlornaphazin	+	Urinary bladder	(*35*)
Chromium		Lung	(*34*)
Cigarette smoke	+	Mouth, larynx, lung	(*36*)
Clonorchis sinensis?		Biliary tract	(*37*)
Diphenylhydantoin		Lymphoma	(*35*)
Estrogens, natural		Endometrium, breast?	(*38, 39*)
Estrogens, synthetic	?	Vagina, cervix	(*40*)
Hematite?		Lung	(*34*)
Immunosuppressants		Lymphoma	(*35*)
Isopropyl alcohol		Nasal cavity	(*34*)
Leather		Nasal cavities, urinary bladder	(*34, 41*)
Melphalan	+	Bone marrow (leukemia)	(*35*)
Nickel		Nasal sinuses, lung	(*34*)
Mustard gas		Respiratory	(*34*)
Phenacetin		Renal pelvis	(*35*)
Radiation, ionizing		Various	(*42*)
Radiation, UV		Skin	(*42*)
Schistosoma haematobium		Urinary bladder	(*37*)
Soots and tars, aromatic hydrocarbons	+	Skin, scrotum, respiratory	(*43*)
Vinyl chloride	W	Liver, angiosarcoma	(*34*)
Wood dust		Nasal cavities	(*34, 41*)

[a] As reported in (*44*). + = mutagenic, 0 = nonmutagenic, W = weakly mutagenic, and ? = chemical toxicity prevented evaluation.

[b] Relatively pure auramine was tested. In industry, impurities may be mutagenic. Magenta contains pararosaniline which is nonmutagenic (McCann J: Personal communication).

Table 2 also shows the results of the Ames test for each of the known human carcinogens: 23 of the 35 either have not or cannot be so tested. Of the remaining 12, 10 were mutagenic, 1 was weakly so, and 1 produced no effect (*44*).

Teratogens That Are Carcinogenic in Man

Only five agents are known to be carcinogenic and teratogenic in man (table 3), but they affect dissimilar organs apparently through dissimilar biologic processes. Thus ionizing radiation, known to cause long-lasting chromosomal breaks, induces leukemia and other cancers (*45*), but its teratogenic effect in man is only on the brain, apparently through cell death (*46, 47*).

TABLE 3.—*Agents that are both carcinogenic and teratogenic*[a]

Agent	Cancer sites	Organs affected
Alcohol	Mouth, larynx, esophagus	Brain, face
Diphenylhydantoin	Lymph glands	Brain, face, nails (hypoplasia)
Ionizing radiation	Various	Brain, stature
Androgens	Liver	Masculinization of the external genitalia of females
Diethylstilbestrol	Cervix or vagina	Cervix and vagina

[a] *See* text and table 2 for references.

The teratogenic effect of alcohol (the fetal alcohol syndrome) is characterized by mental and physical underdevelopment and by hypoplasia of the midportion of the face (*48*). The syndrome is not easily recognized and may be due in part to other environmental exposures of the alcoholic mothers. The carcinogenic effect of alcohol, through an unknown mechanism, is on the mouth, larynx, and esophagus (*32*).

The teratogenic effect of hydantoin is similar but separable from that of alcohol (*49*). A distinctive feature is the tapering of the fingers and absence or hypoplasia of the nails on the fingers and toes—findings that diminish as the child grows older. Case reports suggest that hydantoin, perhaps through its effect on immunity, induces lymphoma, which may be fatal or may regress if administration of the drug is stopped in time (*35*).

Diethylstilbestrol is the only environmental agent reported to date that induces malformations and cancer of the same anatomic site, the vagina or cervix (*40*). The malformations (congenital ridging visible on the surfaces of these organs, or vaginal adenosis) affect a substantial proportion of daughters of women given the drug before the 18th week of pregnancy; susceptibility decreases within that interval as gestational age at first exposure increases (*50*). The carcinogenic effect, which to date has affected fewer than 1 per 1,000 daughters, has the same relation to gestational age as the teratogenic effect does. These observations in man are contrary to the generalization from animal experimentation that malformations occur from exposures during organogenesis, whereas transplacental carcinogenesis occurs from exposures later in gestation, and "do not represent the successive stages of one and the same pathological process" (*51*).

Androgens masculinize female fetuses even when exposure occurs after organogenesis (*52*). The carcinogenic effect of anabolic androgenic steroids is manifested as hepatocellular carcinoma and is virtually limited to patients who received the drug for aplastic anemia (*33*). Liver cancer may be due not to the drug alone (which is widely used by athletes to build up their muscles), but to an interaction of the drug and the disease for which it was given.

OVERLAP OF MUTAGENESIS, CARCINOGENESIS, AND TERATOGENESIS

There is a close correlation between the Ames test of mutagenicity and carcinogenicity in experimental animals (*53, 54*). The overlap is substantially less when all known human carcinogens are considered, in large measure because the test procedure is not appropriate for these agents.

Germinal mutations account for a relation between certain malformations and cancer. These relationships have provided new insights into the carcinogenic process. By contrast, little new insight has yet come from studies of the capacity of a few environmental agents to induce both cancer and congenital malformations. The situation should change with advances in research by clinicians, epidemiologists, and laboratory scientists, if only they would talk to one another.

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ADDENDUM

In the 18 months since the Editorial appeared, no new human carcinogens or teratogens were identified. The Editorial omitted one previously known teratogen, for which additional evidence has recently been published (*1*), i.e., a characteristic facial dysmorphism attributed to trimethadione used (infrequently) for epilepsy during pregnancy (*1–4*). The drug is not known to be carcinogenic.

In May 1977, the estimate of the risk of vaginal–cervical adenocarcinoma among young women after intrauterine exposure to diethylstilbestrol (DES) was refined: 0.14–1.4 per 1,000 (*5*). All fetuses were exposed under 18 weeks of gestational age; i.e., during organogenesis. A high percentage of all women so exposed have minor congenital anomalies of the cervix or vagina, e.g., adenosis or ridging (*6*). Thus DES-related carcinogenesis and teratogenesis appear to have mechanisms in common. Males exposed in utero have an increased frequency of oligospermia, hypotrophic testes, epididymal cysts, and microphallus (*7*), all of which produce trouble enough, but no cancers have been reported. Further problems may lie ahead, as suggested, e.g., by a study in which women exposed to DES during intrauterine life had abnormalities in the shape of the uterus when examined by hysterosalpingogram, and among 7 women so exposed who became pregnant, 1 had a full-term normal infant, 2 had spontaneous abortions, 1 an ectopic pregnancy, and 3 had premature infants (*8*).

Saccharin is another chemical much in the news. Laboratory alarm that this artificial sweetener may be a urinary bladder carcinogen in human beings was not confirmed by a substantial epidemiologic study done in Baltimore (*9*). Saccharin is not known to be a human teratogen.

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Guest Editorial

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Status Report on Saccharin in Humans

BACKGROUND

On the basis of experimental evidence that the artificial sweetener saccharin causes bladder cancer in rodents (*1–6*), on April 15, 1977, the FDA proposed a ban on saccharin. Under existing law, any substance known to cause cancer in animals or man cannot be allowed on the market as a food additive, so the FDA had no choice in its proposed ban. Saccharin is the only nonsugar sweetener on the market, and the public perceives that it benefits from having it available. Because of this perception, the proposed saccharin ban caused a public outcry that was reflected in mail, telegrams, and telephone calls to members of Congress. Congressional hearings on this subject were held in March and June 1977. In December 1977, Congress imposed an 18-month moratorium on the proposed ban so that more information could be collected.

During the hearings questions arose such as: the rationale for equating carcinogenicity in mice, rats, and other lower animals with carcinogenicity in humans; the justification for administering large unphysiologic doses of a material that presumably do not equate to the lower dose used naturally by humans; and the validity of extrapolating the results of large doses in animals to the situation in humans. The answers to these questions relate to the life-spans of the animals, metabolic rates of lower animals compared to those of humans, and the intent of bioassay tests to demonstrate carcinogenicity if it exists rather than one's missing it or attempting to quantify the level of risk.

Answers to these questions are based on the knowledge that all known carcinogens in man (occurring from natural experiments such as occupational exposures) also cause cancer in some lower animal systems. Two possible exceptions are arsenic and benzene. It is not known whether the reverse is true, namely, that substances carcinogenic for lower animals are necessarily carcinogenic for humans. This proposition would be impossible to prove one way or the other at this time. Precise determinations of thresholds for carcinogens are not attainable at the present time, although the evidence is mounting that, as the dose of many carcinogens is increased, additional factors in terms of cancer causation come into play that may be avoidable at lower doses. Also, for some carcinogens evidence suggests that the carcinogenic response is secondary to a primary response that can be prevented by minimizing exposures.

About the same time that the FDA proposed to ban saccharin, a Canadian study of humans suggested that men with bladder cancer were more frequent users of saccharin than were men without bladder cancer; this implies a possible causal association between use of saccharin and bladder cancer (*7*). At least two U.S. studies showed no apparent association between bladder cancer and saccharin use (*8, 9*).

Because of the conflicting results of the human studies, the FDA and the NCI reviewed the existing human epidemiologic studies of saccharin use and bladder cancer. Although NCI has worked with the FDA for years in the area of drug development, this cooperation represented the first time, to our knowledge, that the NCI joined with a regulatory agency to work out a problem that had attracted enormous public and congressional concern.

REVIEW OF EXISTING STUDIES IN HUMANS

Three types of epidemiologic studies were available for in-depth review: two time-trend studies, three studies of diabetics, and three case-control studies. As with almost every epidemiologic study, they all have limitations, many of which are unavoidable.

The following are the limitations of the two time-trend studies (*10, 11*): 1) If a long latent period existed, the effect would not have been manifest; 2) the average doses may have been too small to cause a measurable effect; 3) high doses may have been accumulated in too few individuals to yield effects detected in a correlation of population trends; and 4) the protocol may not have been able to accommodate associated high-risk factors such as cigarette smoking, occupational exposures, and others.

Limitations of the three studies of diabetics (*12–14*)

Abbreviations used: FDA=Food and Drug Administration; NCI=National Cancer Institute; SEER=Surveillance, Epidemiology, and End Results Reporting.

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[2] Environmental Epidemiology Branch, Division of Cancer Cause and Prevention, National Cancer Institute.

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include: 1) failure to ascertain which diabetics were saccharin users and which were not, 2) lack of data for individual exposures to other risk factors, 3) presumption that diabetics as a group were at high exposure without direct measurement of exposure to saccharin, and 4) no determination of level or duration of exposure to saccharin.

These five studies provide some assurance that no epidemic of saccharin-induced bladder cancer has occurred. However, the information they provide cannot establish whether artificial sweeteners have a role in the causation of bladder cancer in humans.

Of the three case-control studies reviewed in detail (*7-9*), two were negative and one was positive. They are not criticized for their basic method, but rather for specific design features (many unavoidable). These features include: 1) Controls selected from a hospital were more likely to be overweight or to have other medical problems than were people chosen at random from the population, and thus perhaps more likely to use artificial sweeteners; 2) low or unequal response rates in case and control series would introduce a bias if respondents were systematically different from nonrespondents, as they often are; 3) history of smoking or occupational exposures and other potentially confounding variables were inadequately controlled; 4) slow identification of cases led to losses prior to interview; 5) the study of all hospitalized cases rather than just those newly diagnosed may lead to an unrepresentative sample of cases of bladder cancer; 6) the dose, timing, and type of exposure were not adequately ascertained; and 7) the numbers of cases and controls were too small to reveal a small increase in risk.

These studies do not provide enough evidence to establish an association between use of artificial sweeteners, specifically saccharin, and an increased risk of bladder cancer in humans. This review culminated in a report submitted to the Commissioner of the FDA by the Interagency Saccharin Working Group and contained a recommendation for the initiation of a new study.

PROPOSED NEW STUDY

The NCI will implement a large-scale population-based, case-control study with the use of cancer-reporting mechanisms of the NCI's SEER[4] program and the State Health Department of New Jersey. The SEER program is a collection of newly diagnosed cancer cases (except skin cancer) in about 10% of the U.S. population (slightly over 20 million people), which represents a wide geographic distribution. In addition to routinely collecting data on incidence, morbidity, and survival, it represents a valuable national resource for studies such as the one we propose. With the state of New Jersey, the SEER areas provide a unique, suitable, and existing resource for such a study. Collaborators will share a common protocol, questionnaire, and operating procedures.

This combination of resources would provide: 1) data on 3,000-4,000 incident cases and 6,000-8,000 controls, which allow for the consideration of potential biasing factors, 2) a wide geographic base, 3) populations that experience a wide range of risks of bladder cancer, 4) a rapid report system for identifying newly diagnosed cases, 5) significant cost reductions through utilization of existing resources, 6) ongoing contracts that can be supplemented in a minimum amount of time, and 7) combined expertise of NCI-FDA epidemiologists, statisticians, and computer capabilities. We plan to begin collecting cases in February 1978 for 12 months. This procedure will allow 4 months for data analyses so that we hope to have some preliminary information by the end of the 18-month congressional action.

In summary, there is not enough evidence to establish an association between use of artificial sweeteners, specifically saccharin, and an increased risk of bladder cancer in humans. The NCI with the FDA is initiating a large-scale study of saccharin use and bladder cancer. This effort is a good example of collaboration by a research institute and a regulatory agency to work on a problem of public importance.

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[4] The study includes four states and six metropolitan areas: Connecticut, Iowa, New Mexico, Utah, Detroit, San Francisco, Oakland, New Orleans, Atlanta, and Seattle.

EDITORIAL

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Drug-Induced Cancer

THE CAPACITY of drugs to cause cancer in human beings has recently gained renewed interest through the report that stilbestrol therapy in pregnant women was associated with the development of vaginal cancer in their daughters (*1*). Knowledge of chemical carcinogenesis has come largely from animal experimentation (*2*) and occupational studies (*3*). The extent to which drugs are known to induce cancer in man is much more limited.

Radioactive drugs.—Radioisotopes represent a special category of drugs in which carcinogenic properties are due to ionizing radiation at the site of deposition in the body. Radioactive phosphorus (^{32}P) is leukemogenic when given to patients with polycythemia vera, a myeloproliferative disorder in which chromosomal anomalies may also contribute to leukemogenesis (*4*). Radium and mesothorium, used in the past for treatment of a variety of illnesses, are bone-seeking isotopes which in dozens of patients have induced osteosarcoma or carcinoma in tissues adjacent to bone, such as the paranasal sinuses (*5*). The same effect has been observed in occupational settings, among radium-dial painters and radium chemists. Thorotrast, once used for the radiographic delineation of blood vessels, localizes primarily in the reticuloendothelial system and in many instances has induced hemangioendothelioma of the liver (*6*).

Immunosuppressive drugs.—Antilymphocyte serum, antimetabolites, and corticosteroids are not true carcinogens, *i.e.*, initiators of malignant disease. However, in patients receiving organ homografts, these agents seem to contribute to a high rate of cancer by suppressing immune defenses that would ordinarily limit the progression of initiated cells. Among survivors of about 5,000 transplants around the world, 52 have developed cancer (*7*). The preponderant neoplasm has been lymphoma (20 cases), half of which showed primary involvement of the brain, usually an extremely rare site of presentation. Because persons with heritable immune-deficiency disorders are prone to lymphoma and possibly other cancers (*8*), immunosuppressive agents are probably involved in oncogenesis following transplantation. Additional factors may be necessary, since there is no evidence that cancers develop excessively in other conditions treated with immunosuppresive drugs. An interplay may exist between immunosuppression and special characteristics of the transplant patient (*e.g.*, chronic antigenic stimulation by the homograft) to create a state highly susceptible to cancer.

Other drugs.—Few drugs are well-documented chemical carcinogens in man. *Chlornaphazine*, used in the treatment of polycythemia vera and Hodgkin's disease, was withdrawn in 1964 when high doses were found to cause bladder tumors (*9*). A recent follow-up in Denmark of 61 treated patients revealed 10 with bladder tumors and 5 others with atypical cells in the urine (*10*). The

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oncogenic capacity of the agent may have been predicted, since it is a derivative of β-naphthylamine, a bladder carcinogen in industrial workers. *Alkylating agents*, also used primarily in the treatment of cancer, are nearly always carcinogenic in laboratory animals (*2*). Accumulating evidence in man suggests that certain agents are causally related to acute leukemia, especially the myelogenous or monomyelogenous types. Most striking has been an increase in acute leukemia reported among multiple myeloma patients treated with melphalan or cyclophosphamide (*11*). In these instances it is not known whether the drug causes the neoplasm, or whether prolonged survival allows time for development of a cancer that is part of the underlying disease for which the drug was used. *Arsenic* has not exhibited carcinogenicity in experimental animals. However, when taken internally for a prolonged time, inorganic arsenicals are generally recognized as a cause of skin cancer in man (*12*). The cancers are usually multiple, involve unexposed parts of the body and unusual locations such as the palms of the hand, and are associated with skin pigmentation and hyperkeratosis. Cancers of the lung have been implicated after heavy occupational exposure to arsenic, but the evidence for a causal relation is less secure (*13*).

Far less conclusive is the evidence that other drugs are carcinogenic in man. Heavy consumption of analgesic drugs containing *phenacetin* is a causal factor in chronic pyelonephritis and papillary necrosis. Reports from Sweden indicate that patients with this condition, termed "analgesic nephropathy," are at high risk of developing transitional cell tumors of the renal pelvis (*14*). The complication has not been reported in other countries, but, in view of the continued widespread use of analgesic mixtures containing phenacetin, studies are urgently needed to fully evaluate this hazard. *Diphenylhydantoin* (Dilantin) is effective in the treatment of epilepsy and occasionally induces lymph node reactions that regress on cessation of therapy. Transformation of this state to malignant lymphoma has been reported in several patients, but the nature of this association is ill defined (*15*).

Coal tar and creosote preparations in dermatologic practice contain polycyclic hydrocarbons that produce skin cancer in laboratory animals and men who are occupationally exposed. In a few reported cases, medicinal application also seemed to cause skin cancer (*16*). Polycyclic hydrocarbons were once present in *liquid paraffin* (mineral oil), commonly used as a laxative. In a retrospective study, a relation was suggested between gastrointestinal cancer and heavy use of this drug (*17*), but was not confirmed by an equally well-executed study (*18*). In addition, case reports have implicated various marrow-depressing drugs, particularly *chloramphenicol*, in the development of leukemia (*19*, *20*). A causal relationship would be consistent with the potential of other agents (radiation, benzene, alkylating agents) to produce aplastic anemia and leukemia (*20*, *21*). Chromosomal defects caused by chloramphenicol are noteworthy, since the inborn and acquired conditions at high risk of leukemia have cytogenetic abnormalities (*21*).

Among the best evidence for a carcinogenic drug in humans is the recently described link between *stilbestrol*, a synthetic estrogen, and a time-space cluster of vaginal adenocarcinoma in 8 women between 14 and 22 years of age (*1*). The usual rarity of this tumor in young women drew attention to the cluster and prompted a retrospective study showing that the mothers of 7 of the 8 girls were treated with stilbestrol during pregnancy to prevent miscarriage. If the same increase in frequency had occurred for a more common tumor, it would likely have gone undetected. This first demonstration of transplacental carcinogenesis in women opens new vistas concerning *in utero* exposures, and appears to be a human counterpart to the heightened susceptibility of newborn animals (*22*) and embryos (*23*) to experimental carcinogenesis. To date, there is no evidence in humans that estrogens given after birth induce cancer. Tumors of the breast, reported to be excessive in men receiving stilbestrol for prostatic cancer, now appear not to have been primary in the breast, but metastases from the prostate (*24*). However, the stilbestrol episode and the capacity of estrogens to induce tumors in experimental animals (*25*) point to the need for the careful surveillance of groups of patients treated with estrogens.

The future.—The evidence for occupational cancer is considerably more extensive than that for drug-induced cancer in man (table 1). Why is this so? In contrast to animal studies, it may well be that in man the doses are too small or too briefly given, the latent period for carcinogenesis is longer than the interval of observation, or confounding variables prevent easy recognition of relationships.

With these considerations in mind, future investigations of drug-induced cancer may be pursued on many fronts. 1) Animal and other laboratory studies can suggest human responses and should be expanded, particularly in regard to quick screening procedures for premarketing tests. 2) Surveillance of trends in human cancer may reveal peaks by cell type, age, sex, and locale. Earlier analyses by cancer registries should enable prompt detection of patterns that might subsequently be related to drug exposure. 3) The alert clinician may provide important clues to environmental agents by reporting clusters of cancers that are rare in the general population, particularly when the series has unusual demographic, clinical, occupational, familial, or histopathologic characteristics. A specific drug relationship may be suggested by the association of cancer with known toxic manifestations, such as arsenical dermatoses. Consultation should be available to such practitioners by the National Cancer Institute and the International Agency for Research on Cancer, so that clinical leads will be utilized to the fullest. 4) Retrospective studies can identify previously unsuspected carcinogenic drugs, as in stilbestrol-induced cancers of the vagina. 5) Prospective studies, effective in confirming drug–cancer relationships, are expensive and time consuming and best used when other approaches suggest a possible hazard. This method was applied, for instance, to persons receiving *isoniazid*, carcinogenic to laboratory animals, but reports to date indicate no increased risk of cancer (*26*). 6) Special disease registries may contribute to the detection of drug-related cancers, as in collected series of patients with organ transplants, connective-tissue disorders, adverse drug reactions, multiple primary tumors, or cancers evaluated by cooperative clinical trials. 7) Record-linkage systems are promising as effective and economical monitors of drug-induced cancer. For example, methods to link drug prescriptions with subsequent diagnoses of disease have been instituted as a surveillance system for drug toxicity at Kaiser-Permanente in San Francisco (*27*). Prepaid health insurance plans, such as that at Kaiser-Permanente, have large samples of patients followed over time, a considerable advantage in longitudinal studies to evaluate the risk of cancer and other long-term effects related to drug exposure. 8) Evaluation of *in utero* or childhood exposures to drugs should be emphasized in future studies on laboratory animals and man.

TABLE 1.—Cancers due to occupational exposures, as compared with drug induction

Occupational chemical carcinogenesis (*3*)		Drug-induced cancer*	
Asbestos	Lung Mesothelium	Radioactive drugs	Organs where concentrated
Nickel	Lung Paranasal sinuses	Immunosuppressive drugs	Lymphoma (other?)
Chromates	Lung	Chlornaphazine	Urinary bladder
Arsenic	Lung (?) Skin	Melphalan Cyclophosphamide	Acute myelomonocytic leukemia (?)
Mustard gas	Respiratory tract	Arsenic	Skin
Coke-ovens	Lung	Phenacetin-containing drugs	Renal pelvis
Woodwork	Nasopharynx	Diphenylhydantoin	Lymphoma (?)
Chimney sweeps Tar workers Cotton spinners	Skin Scrotum	Coal-tar ointments	Skin
α- or β-Naphthylamine	Urinary bladder	Chloramphenicol	Leukemia (?)
Benzidene	Urinary bladder	Prenatal synthetic estrogens	Vagina
4-Aminobiphenyl	Urinary bladder		
Benzene	Leukemia		

**See* text for references.

To give fresh impetus to the study of drug-induced cancer, new lines of communication are needed between clinicians, experimentalists, and epidemiologists.

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ADDENDUM

The major discovery that diethylstilbestrol (DES) is a transplacental carcinogen led to a search around the world for cases of vaginal and cervical clear cell carcinomas in young women. From 1970 to 1976, details of over 333 cases in females ranging from 7 to 27 years of age, with a peak at age 19, were compiled by Herbst and his colleagues (*1*). A history of prenatal exposure to DES or other nonsteroidal estrogens was found in about two-thirds of them. The estimated attack rate of clear cell carcinomas following in utero exposure has been less than 1/1,000. In contrast, a high proportion of exposed women have shown benign alterations (e.g., adenosis and transverse ridges) of the vagina and cervix (*2*). Some have had adjacent areas of squamous metaplasia and dysplasia, raising concern about possible progression to squamous cell carcinoma.

The carcinogenic potential of prenatal exposure to DES stimulated several investigators to evaluate the effects of this drug given after birth. In 1972, an unusual form of endometrial carcinoma (mixed or adenosquamous type) was reported in a series of young patients with gonadal dysgenesis treated with DES for at least 5 years (*3*). A follow-up survey of women with breast cancer confirmed an increased risk of endometrial cancer, with the excess limited to those treated with hormones, primarily DES (*4*). A recent follow-up of *mothers* who took DES to prevent complications of pregnancy revealed some increase in their risk of breast and ovarian cancers compared with an untreated control group, although the differences were not considered significant (*5*).

Another major development occurred in 1975, when 2 case–control studies linked endometrial cancer to the use of conjugated estrogens for menopausal symptoms (*6, 7*). In several reports, the relative risks of this cancer have varied from fourfold to ninefold, with a gradient associated with the strength of medication and duration of use (*8*). The rising incidence of endometrial cancer during the 1970's in the United States has been related to the use of estrogenic preparations by women during menopause (*9*). Furthermore, in a recent follow-up study of women receiving menopausal estrogens, the risk for breast cancer was 1.3 times the expected value, rising to twofold 15 years after initial exposure (*10*). The risk of ovarian cancer was two to three times expectation but affected only a small group of women taking DES and conjugated estrogens (*11*).

Suspicions about the carcinogenic hazards of oral contraceptives were heightened in 1973 when several exposed women were reported with benign liver tumors (*12*). These were often life-threatening lesions due to sudden rupture and hemorrhage. Two case–control studies of hepatocellular adenoma established an increased risk associated with long-term use of oral contraceptives, especially among women over 30 years of age taking pills containing high doses of steroids (*13, 14*). Although the evidence is incomplete, oral contraceptives of the sequential type have also been related to endometrial carcinoma. In a series of 30 patients under 40 years of age with a history of oral contraceptive use, the proportionate exposure to sequential versus combination agents was much higher than expected in the general population (*15*). No clear-cut evidence links oral contraceptives to breast cancer, but some studies have suggested a hazard for high-risk women, e.g., those with cystic mastopathy, late age at first birth, or a family history of breast cancer (*16, 17*). Recent reports have also related use of oral contraceptives to carcinoma-in-situ of the cervix, cutaneous melanoma, and pituitary adenoma, but the data are insufficient to draw conclusions at this time (*8*).

Androgenic–anabolic steroids in the form of oxymetholone or methyltestosterone derivatives were linked to hepatocellular carcinoma by a series of case reports of patients undergoing long-term therapy for aplastic anemia (*18*). Cases with Fanconi's anemia seemed at special risk, in accord with their existing predisposition to acute leukemia and other cancers. However, the liver tumors arose when the agents were given for conditions other than aplastic anemia and have regressed sometimes on drug withdrawal (*19*). Another hormonal agent was brought to attention by a report relating breast cancer to the use of thyroid supplements for hypothyroidism (*20*). This study is inconclusive, however, due to difficulties in methodology and in distinguishing the effects of therapy from the underlying thyroid disease.

An increased risk of leukemia was established in patients with multiple myeloma and Hodgkin's disease who were treated with alkylating agents (*21*). However, in these particular conditions, it seems possible that longer survival has permitted expression of an intrinsic predisposition to leukemic transformation. To estimate better the leukemogenic potential of cytotoxic drugs, Reimer et al. (*22*) studied 5,455 patients with ovarian cancer from several institutions. Thirteen developed acute nonlymphocytic leukemia, and all had received alkylating agents. The risk of leukemia in the patients given chemotherapy was 36 times the expected value, rising to 171 in those surviving 2 years. This leukemogenic potential may be acceptable when treating conditions with a poor prognosis, such as breast cancer with axillary node involvement, but further consideration is needed before using the agents for conditions with a more favorable survival rate.

Immunosuppressive agents have been assessed primarily by follow-up studies of recipients of renal transplants (*23*). Among 16,290 transplant patients registered to date, the risk of non-Hodgkin's lymphoma was 32-fold and mostly of the histiocytic type. Many cases developed within months of transplantation, which suggested a breakdown of immunosurveillance with loss of control over lymphoid cells already initiated and transformed. For other tumors combined, the excess risk was about twofold and first evident about 2 years after transplantation. It has not affected all tumors across-the-board, as might be predicted by the immunosurveillance hypothesis; instead, increases in the risks were modest for cancers of the liver, bile duct, and bladder, plus lung adenocarcinoma, leukemia, soft-tissue sarcoma, and skin cancers including melanoma. Immunologic mechanisms may be involved in some instances, as suggested by a recent survey of patients with chronic lymphocytic leukemia that indicated an increased risk of melanoma, soft-tissue sarcoma, and lung cancer (*24*).

The DES episode prompted efforts to identify in utero exposures to other agents in tumors of young people. Diphenylhydantoin is a suspect drug, since neuroblastoma has been reported in 2 children and malignant mesenchymoma in 1 child, with congenital defects attributed to it (*25*). A recent case–control study of children with brain tumors suggested an excess risk after maternal use of barbiturates during pregnancy and following use during early childhood (*26*). An earlier follow-up of adult patients treated for epilepsy with phenobarbital, diphenylhydantoin, or primidone revealed an elevated risk for cancers of the brain and nervous system; however, these tumors were thought by the authors to have caused the seizures in the first place (*27*). In this context, it may be relevant that in mice, phenobarbital enhances the development of liver tumors induced by various carcinogens and seems to increase the rate of spontaneous liver tumors as well (*28*).

Reserpine was suggested as a risk factor for breast cancer by 3 studies reported in 1974. More recent investigations, however, indicate that the relationship may be explained by social class and other confounding variables (*29, 30*). An uncontrolled case series raised suspicions about another antihypertensive drug, spironolactone (*31*), but the association with breast cancer has not been confirmed (*32*). Several patients have been reported with sarcomas arising at sites of previous iron–dextran injections (*33, 34*).

Although these preparations have induced sarcomas in laboratory animals, the risk in man appears to be extremely small.

Thus recent epidemiologic observations indicate that the carcinogenic hazards of estrogenic and other medicinal agents are greater than previously thought. Further research along the lines suggested by our 1972 Editorial and subsequent reviews (*35, 36*) should lead to improvements in the recognition and control of drug-associated cancers.

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GUEST EDITORIAL

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Exogenous Hormones—Boon or Culprit?

EVER SINCE the classical work of Beatson in 1896 (*1*) and Loeb who reported in 1941 (*2*) on his work done in his earlier years, there has been a steady scientific and public debate as to the role of estrogens in the development of human cancer. In this debate, clinical evidence in terms of hormone-related cancers was often translated as hormones being a "cause" of such cancers.

Single case reports of cancers of the breast or prostate developing after the application of relatively high doses of estrogens were considered by some to indicate that hormones administered in physiologic doses could be carcinogenic to man. Similar conclusions were drawn from studies showing that estrogens could induce breast cancer in animals susceptible to mammary cancer. Some studies have suggested that a given hormonal milieu protects or makes an individual more susceptible to hormone-related cancers (*3*, *4*). There has been no clear-cut agreement on this issue, however.

Women using contraceptive pills seem to have about the expected rate of breast cancer, whereas they less commonly develop benign diseases of the breast (*5*, *6*). Several studies (table 1) have suggested that long-term estrogen therapy can either reduce or delay the onset of breast cancer (*7–14*).

If one were to take an opinion poll of scientists and the lay public, one would find that the majority of both groups consider estrogens to be carcinogenic to humans. We suggest that estrogens at least when given in physiologic doses, have not been established as being carcinogenic to women. We recommend that studies be carried out to elucidate this important public health question. The effect of birth control medication is now being widely studied (one of these studies being conducted by our group). The end results are difficult to obtain because relatively few women take the pill for prolonged periods. Of even greater interest would be a study of women who are on prolonged hormonal therapy throughout the climacteric period and, particularly, throughout their postmenopausal life.

Within the confines of this statement, we shall not discuss the many epidemiologic problems involved in the studies suggesting reduced risk of breast cancer among women on long-term hormonal therapy. However, we are sufficiently impressed with these data to recommend additional efforts. If physicians treating patients in this manner were to pool their resources, we

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

TABLE 1.—*Incidence of breast and gynecologic cancer after long-term administration of estrogens*

Authors (yr)	Number of patients	Age (yr)	Reasons for estrogen administration	Methods and duration of administration	Follow-up (yr)	Cancer incidence	
						Site	Observed/ expected
Geist et al. (1941).	206	25–80	Menopausal syndrome, functional amenorrhea, senile vaginitis, kraurosis vulvae.	Estrogens only, 6 months–5.5 yr.	1–5	Breast & gynecologic	0/12
Henneman et al. (1957).	292	15–83 (avg. 51).	Menopausal syndrome, postmenopausal, osteoporosis.	Cyclic estrogens, >1 yr (avg. 5.1 yr).	1–25	Gynecologic (cervix 1, ovary 2, endometrium 4).	7/22
Mustacchi and Gordon (1958).	120	15–84 (avg. 62).	Osteoporosis	Estrogens, 3–8 yr, alone, androgens, or estrogens+androgens.	>1	Breast & gynecologic	0/5–6*
Wilson (1962)	304	40–70 (avg. 51).	Estrogen-deficient women according to Pap smear test.	Cyclic estrogens, 1-27 yr.	1–27	Breast & gynecologic	0/18
Schleyer-Saunders (1960).	300	30–60	Artificial menopause, osteoporosis, senile vaginitis, etc.	Estrogen/androgen & deoxycorticosterone. Combination pellet. Several years.	Several	Breast 3 & gynecologic 4 (cervix 1, ovary 2, endometrium 1).	
Leis (1966)	158	36–54 (avg. 46).	Menopausal syndrome, including some cases of artificial menopause.	Cyclic estrogens 8 yr; progesterone, 10–14 yr.	8–14	Breast & gynecologic	0/unknown
Burch and Byrd (1971).	511	27–72 (avg. 54).	Hysterectomy	Estrogens only, long term.	29	Breast	9/39*

*Expected number for all malignant tumors.

believe a meaningful investigation could be planned and completed within 3 years. Obviously, a carefully selected control group would be an essential part of such a study. We would be prepared to coordinate such a study and would appreciate hearing from all physicians who have records of women receiving long-term estrogen therapy for any known period and who are willing to have *all* these cases carefully followed by our staff. Such a study should be able to answer the intriguing question: "Are exogenous estrogens, when given in physiologic doses, a boon or a bane with respect to the subsequent development of cancer in women?"

If the long-term administration of estrogens at a physiologic level enhances or reduces the risk of women for developing cancer of the breast and/or other cancers, we certainly should be aware of it as soon as possible so that physicians can act accordingly. No change in risk would indicate the lack of demonstrable carcinogenicity of the hormones.

In either case, we welcome the cooperation of the scientific community in helping us solve this vital public health issue.

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GUEST EDITORIAL———————by **W. M. Christopherson, *M.D.*,**[1] *and* **E. Truman Mays, *M.D.***[2]

JNCI 58:167–171, 1977

Liver Tumors and Contraceptive Steroids: Experience With the First One Hundred Registry Patients

Oral contraceptives have recently been implicated in the development of benign liver cell tumors in young women. The magnitude of the problem is as yet not known, but with an estimated thirty-five million American women who either take or have taken "the pill," the potential is evident.

Baum et al. (*1*) were the first to suggest the role of contraceptive steroids in benign hepatomas. Four of the seven patients they described had hemoperitoneum as the result of tumor rupture. Davis et al. (*2*) had previously reported massive hemoperitoneum from rupture of hepatocellular adenomas. All three of their patients were young women, one of whom was pregnant. The authors did not, however, include the oral contraceptive histories in their publication. Mays et al. (*3*) described four patients with FNH. All were taking oral contraceptives; two of the patients had hemoperitoneum and one a large intrahepatic hematoma. These authors implicated the vascular lesions within the regenerating liver nodules, postulating that occlusive lesions of the afferent vessels, possibly secondary to oral contraceptive ingestion, might play a role in the pathogenesis.

Soon after the publication of Baum et al., additional reports began to appear, and by March 1976 an estimated 107 cases had been reported (*4*). Since then, several additional reports have appeared, one included 22 new cases (*5*).

Meyer et al. (*6*) reported a hepatoblastoma in a 19-year-old woman on oral contraceptives. This was the first and only report of such a tumor associated with oral contraceptives. It should be noted that hepatoblastomas ordinarily occur in infancy or early childhood. O'Sullivan and Wilding (*7*) reported three patients with hamartoma of the liver; each had a history of contraceptive ingestion. Christopherson et al. (*8*) reported and illustrated a liver cell carcinoma with extensive vein invasion occuring in a woman on oral contraceptives.

In late 1973, we began a registry of liver tumors at the University of Louisville. The purpose was to provide a repository for clinical and pathologic data on these tumors, as well as to record drug association. The following is a brief resumé of the first one hundred patients accessioned in the registry. Several of the cases have previously been reported.

RESUMÉ OF CLINICAL FINDINGS

Patients with benign tumors varied in age from 14 to 47 years. One patient with a hepatoma and user of conjugated estrogens was 51 years old.

The age distribution and average age are shown in table 1. The average age for all patients was 29.8 years.

Table 2 shows the type of estrogen and includes other factors that may be related. The history was incomplete in five instances and the specific pill was not recorded in nineteen additional patients. Three patients with adenoma had never taken oral contraceptives; two were age 15, the other 14.

A fourth patient with streak ovaries who had not taken oral contraceptives had a malignant hepatoma (henceforth referred to as hepatoma). One patient with hepatoma had Stein-Leventhal syndrome, and the tumor was discovered at time of wedge resection of her ovaries. The patient had ingested oral contraceptives in an attempt to regulate bleeding.

Of particular interest were three women who were long-term users of conjugated, equine estrogens; an additional one with a thecoma and six patients who were pregnant or immediately post partum.

The women on oral contraceptives tended to be long-term users. The length of exposure, however, ranged from 4 to 156 months (table 3).

The pattern of the type of progestin was a random one with seven different compounds represented, often in combination. As would be expected, norethnodrone was most commonly used since it is the principle progestin utilized in oral contraceptives and has been available since at least 1963. Norethnodrel, the second most commonly used progestin in this group, while a component of relatively few oral contraceptives, has been available since 1960.

Clinical Presentation

Pain and hemoperitoneum were the most common clinical findings. Adenomas bled more frequently than did FNH, while the latter was more apt to be an incidental finding (table 4). Ruptured ectopic pregnancy was the most frequent preoperative diagnosis in patients with hemoperitoneum. A diagnosis of peptic ulcer or gallbladder disease was often made in women with pain

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Abbreviation used: FNH = focal nodular hyperplasia(s) of the liver.

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TABLE 1.—*Age distribution and average age, yr*

Tumor type	<20	20–24	25–29	30–34	35–39	40–44	45–49	50–54	Unknown	Total	Average age
Adenoma	4	3	11	13	6	1	1	0	1	40	29.5
FNH	2	7	14	11	4	1	3	0	2	44[a]	29.8
Hepatoma	1	4	3	1	1	1	1	1	0	13	30.4
Unclassified	0	0	2	1	1	0	0	0	0	4	31.0
Total	7	14	30	26	12	3	5	1	3	101	29.8

[a] For one patient, first tumor occurred at age 25, second at age 31.

TABLE 2.—*Number of tumors in women according to different treatments and parameters*

Type of estrogen and other possible related factors	Adenoma	FNH	Hepatoma	Unclassified	Total
Mestranol	15	23	4	1	43
Ethinyl estradiol	3	1	3	1	8
Mestranol and ethinyl estradiol	6	5	1	0	12
Premarin	0	2	1	0	3
Thecoma	0	1	0	0	1
Pregnancy	0	1	1	0	2
Post partum	3	0	1	0	4
Never on pill	3	0	1[a]	0	4
Pill type unknown	8	8	1	2	19
History not known	2	3	0	0	5
Total	40	44	13	4	101[b]

[a] Patient with streak ovaries.
[b] One patient with second tumor.

but without shock, syncope, anemia, or other signs of hemorrhage. Primary or metastatic liver tumor, liver cyst, and liver abscess were the favored diagnosis when a mass was demonstrated. It is of particular interest that three patients were thought to have an ovarian cyst or ovarian tumor. Many of the patients in whom the diagnosis was incidental to laparotomy were undergoing pelvic surgery.

Laboratory and Radiologic Findings

The liver function tests were usually within normal limits. An elevated level of alkaline phosphatase was present in several patients and actually led to liver scan with resultant diagnosis in at least one patient. Other liver enzymes were variable. Liver scan and selective arteriogram contributed to the preoperative diagnosis in several patients and proved of great value in patient follow-up. An occasional tumor was suspected from routine gastrointestinal films.

RESUMÉ OF PATHOLOGIC FINDINGS

Insofar as could be ascertained, there was neither historic nor morphologic evidence of cirrhosis or of hepatic fibrosis in the residual liver of any of these patients, including those with hepatoma.

One patient had FNH resected at age 25 only to have a second similar tumor in her residual lobe at age 31. In the interim she had continued on oral contraceptives. An additional patient had a history of having had prior rupture of FNH. She too had continued her oral contraceptives.

The majority of the tumors were solitary; however, eight patients with adenoma and a similar number with FNH had multiple simultaneous tumors. The hepatomas were usually multifocal (fig. 1); however, in three of the patients they were apparently solitary. The right lobe was most commonly involved. Necrosis and hemorrhage within the tumor were common even in tumors that had not produced hemoperitoneum (fig. 2). Adenomas were more often hemorrhagic than FNH (fig. 3).

We were unable to classify four tumors. One of these underwent total necrosis following hepatic artery ligation 2 months prior to resection. Another tumor, while cytologically malignant, had no vein invasion, and the follow-up was short-term. In two other cases there was inconclusive diagnosis because of limited pathologic material. For a detailed description of the histopathologic aspects the reader is referred to the fascicle on tumors of the liver by Edmonson (*9*).

TREATMENT

The most common treatment was either lobectomy, partial resection of the liver, or resection of the tumor. Twelve patients with benign tumors had biopsy only. In eight of the thirteen patients with hepatoma, no attempt was made to resect the lesion. One hepatoma was first diagnosed at autopsy, as was one FNH. Death related to the disease or to treatment occurred in three patients with benign tumors and in nine with hepatoma. Of the remaining four patients with hepatoma, two are known to have metastasis, one is living 6 months post resection, and the vital status of the other patient is unknown.

When rupture with hemoperitoneum was present, hemostasis was often difficult. Ligation of the hepatic artery was a life-saving procedure in several patients, and resulted in apparent self-destruction of the tumor in at least one other patient.

COMMENT

The recently observed abrupt increase in benign liver tumors in young women suggests the possibility of an environmentally related factor. The increased usage of contraceptive steroids since they were introduced in 1960 and their association with these tumors suggests a causal relationship. At the same time, the large number of women using this medication makes the relationship difficult to prove. In this series, the first case occured in 1966. Only two or three cases occured in any one year until 1972. Over half were diagnosed in 1974 and 1975. This is not surprising since the registry was not started

TABLE 3.—*Duration on pill, mo*

Type of tumor	1–12	13–24	25–36	37–48	49–60	61–72	73+	Unknown	Total
Adenoma	1	3	2	1	4	1	18	3	33
FNH	4	3	3	4	5	3	12	4	38
Hepatoma	3	2	0	1	1	0	2	0	9
Unclassified	0	0	0	1	1	0	2	0	4
Total	8	8	5	7	11	4	34	7	84

TABLE 4.—*Presenting symptoms and signs*

Type of tumor	Hemoperitoneum	Pain	Mass	Incidental	Not recorded	Total
Adenoma	16	12	9	2	1	40
FNH	6	14	7	16	1	44
Hepatoma	1	5	3	2	2	13
Unclassified	3	1	0	0	0	4
Total	26	32	19	20	4	101

until late 1973. However, the previous rarity of benign liver tumors is well documented (*9–11*). An estimated thirty-five million American women have taken or are taking oral contraceptives (*4*). There are even estimates of as high as fifty million (*5*). Although the magnitude of the problem is still unknown, present evidence indicates that it is relatively small in relation to the number of women using contraceptive steroids.

The estrogen rather than the progestin component has been the major suspect. It has even been suggested that mestranol is a greater determinant of risk than ethinyl estradiol (*12*). There is some evidence to show that the risk increases with duration of ingestion. Since mestranol was exclusively used prior to 1964 and at least in the earlier years was the most commonly used estrogen, we would reserve judgment on this point.

The progestins utilized in oral contraceptives have a close structural relationship to anabolic steroids, and androgenic anabolic steroid therapy has been associated with hepatocellular carcinoma (*13, 14*). One tumor submitted to the registry, but not included in this series, occurred in an orchiectomized male who had received testosterone over a number of years. Multiple tumor masses were found in a noncirrhotic liver and the diagnosis of hepatoma was made. Only a biopsy was performed. That the patient is alive and well 15 years later casts some doubt on the diagnosis. It should also be noted that the other androgenic steroid-associated tumors did not metastasize.

The presence of liver tumors in three women using conjugated equine estrogens and in six additional patients who were either in late pregnancy or the early postpartum period as well as one patient with a feminizing ovarian tumor suggests that estrogens may be the predominant factor.

If estrogen potency in oral contraceptives is related to risk, lesser amounts of estrogen in contraceptive pills would seem to be desirable. Oral contraceptives with estrogen content of less than 50 μg have recently been introduced. Estrogen potency of oral contraceptives is said to depend not only on the type and dose of estrogen but on the progestin as well. Hence a pill with lesser amounts of estrogen might not have decreased estrogen potency (*15*). Ethinyl estradiol has considerably greater estrogenic potency than does mestranol (*16*) which again raises the question as to whether the observed excess usage of mestranol over ethinyl estradiol in liver tumor patients is significant (*12*).

If estrogens are involved in oncogenesis, are they initiators, co-oncogens, or promoters? Unfortunately, this is not known. It would seem reasonable, however, that liver tumors, like other tumors, have a multifactoral etiology and that sex steroids play a limited, albeit important, role. Our own attempts to produce liver tumors in young female mice by feeding contraceptive steroids over many months have thus far been unsuccessful. Others, however, have had some success in producing liver tumors in female rats by use of synthetic sex hormones (*17*).

The liver contains enzymes that degrade estradiol, the primary and most active estrogen, to estrogens of lower activity or to inactive substances. In patients with liver disease, notably cirrhosis, there is diminished activity of the liver in removing estrogen from the blood stream and in destroying or conjugating it (*18*).

Patients with hepatoma usually have cirrhosis. An 89% incidence of cirrhosis has been recorded in hepatoma patients (*19*). Clinical manifestations of estrogen activity may be present in patients with cirrhosis; these include menstrual problems, infertility, and breast changes in women, and gynecomastia, testicular atrophy, decreased libido, impotence, and infertility in males. This suggests to us that estrogens could play a role in the development of hepatomas in patients with cirrhosis. This possibility is at least worthy of further investigation. It is noteworthy that in the registry group no patient, including the thirteen with hepatoma, had historic or morphologic evidence of antecedent liver disease including cirrhosis or even liver fibrosis.

The vasculitis, organizing thrombi, and occlusive vascular disease associated with FNH raises the possibility that oral contraceptives could play a role in its development (*3*). On the other hand, it might be speculated that these obstructive vascular changes only contribute to ischemia and eventual necrosis, hemorrhage, and rupture of liver tumors.

Since liver tumors may present in such a catastrophic manner, they are often first seen by the obstetrician–gynecologist, since the symptoms closely mimick those of ruptured ectopic pregnancy. When massive bleeding occurs, ligation of the hepatic artery may be a life-saving procedure. After hepatic artery ligation, elective resection can be carried out at a more opportune time.

The question of the necessity of removing a benign liver mass has arisen on several occasions. This usually arises when multiple, inaccessible tumors are present. In one registry case, the adenoma had ruptured and only a biopsy was performed. A tubal ligation was subsequently done and the patient taken off oral contraceptives. She is alive and well 10 years later without further surgery. There are twelve patients in which it is known that tumor was left behind. They will be followed with great interest. It would seem advisable that sex hormones should be withheld from any patient with a prior liver tumor problem and certainly for those in whom the tumor was not resected.

In summary, there seems to be reasonable evidence that contraceptive steroids are involved in the sudden increase of benign liver tumors in young women. The evidence is less compelling in the case of hepatoma, a more commonly encountered neoplasm. Through the accession of such cases in this and other registries, a more rapid and lucid evaluation of the problem should evolve. All drugs have their adverse reactions and none is without risk—oral contraceptives are no exception. At this point in time however, the risk of liver tumors in oral contraceptive users appears to be very small and should be evaluated in terms of the benefit of the medication. A logical regimen would seem to be careful surveillance of contraceptive users for this rarely encountered event.

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ADDENDUM

Since the Guest Editorial in February 1977, well over 100 additional cases have been added to the registry. Histopathologic studies of some steroid-related adenomas suggest that they may represent monomorphic hepatocytic hyperplasia and in the true sense may not be neoplastic. This is especially true of small tumors. Others are encapsulated and have an adenomatous arrangement of the hepatocytes. Most of the liver nodules in experimental animals have no capsule and lack bile duct proliferation. From the standpoint of pathogenesis, focal nodular hyperplasia and adenoma may be similar (*1*).

Our observation that some tumors regress once oral contraceptive usage is discontinued has been reinforced by additional examples in the registry and reports of others. On the other hand, a second tumor has appeared in another patient who continued to use steroidal medication.

In the registry, 20 hepatocellular carcinomas occurred in patients at a mean age of 25. All were in noncirrhotic livers. Seventeen patients had used oral contraceptives, 2 were either pregnant or postpartum and 1 denied usage. Unlike the "hepatomas" that occur in males who ingest androgenic anabolic steroids, these tumors metastasize and produce death. Sixteen of the 20 patients are known to be dead; of these, 11 are known to have had metastatic spread.

Finally, α_1-antitrypsin was a protein marker for oral contraceptive-associated hepatic tumors of all types; also, α_1-antitrypsin phenotyping of several patients was normal, i.e., of the MM type (*2*).

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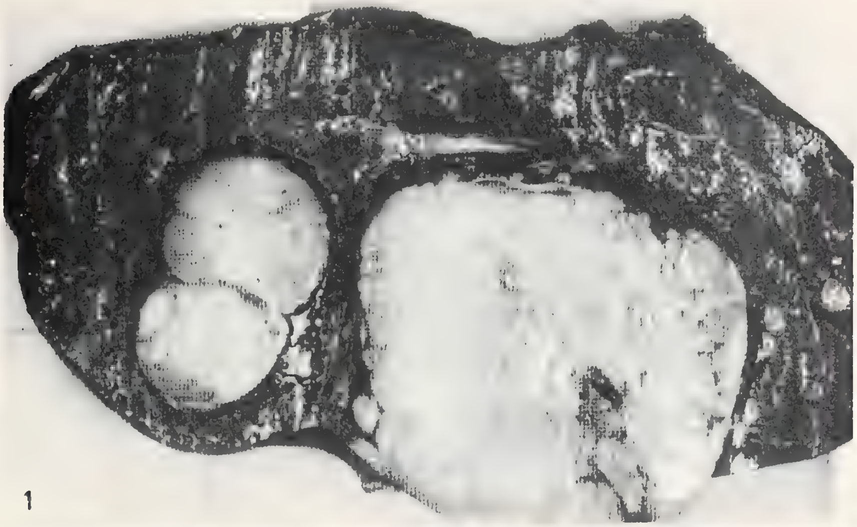
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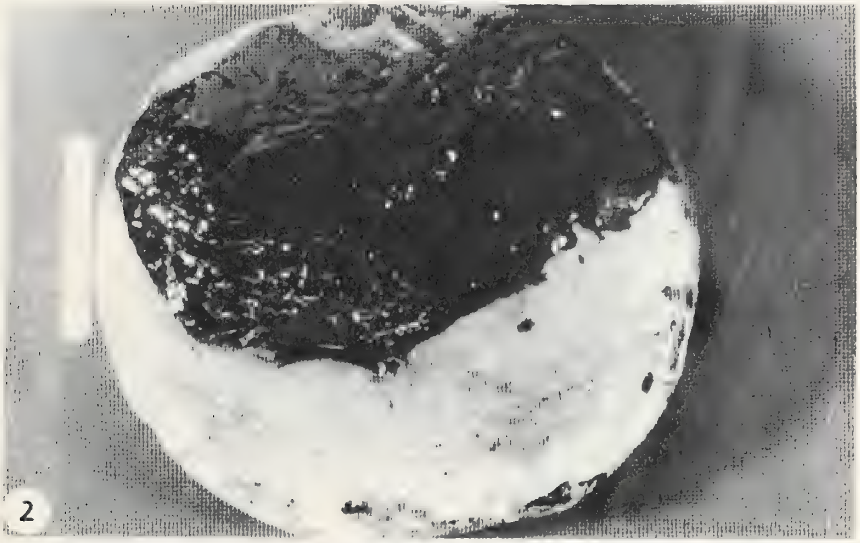
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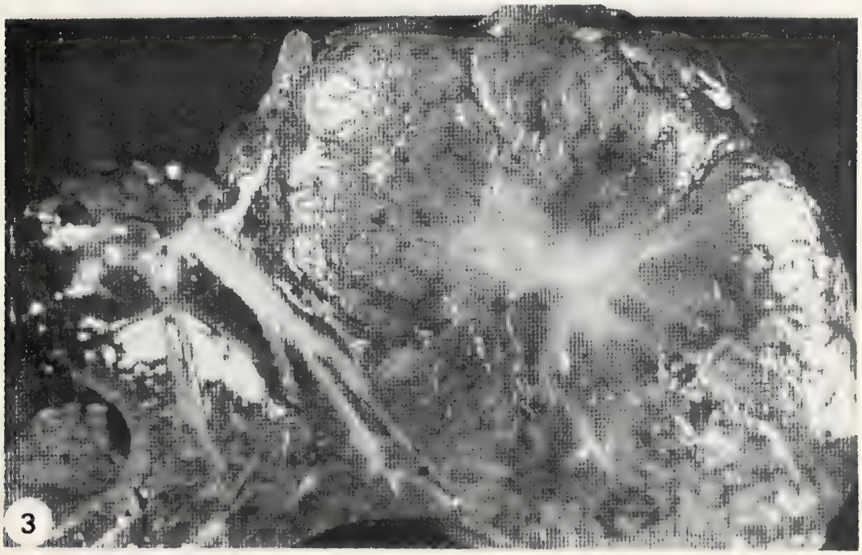
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Figure 1.—Hepatoma, resected specimen, several wk after rupture and hemoperitoneum. *Note* small tumor thrombi in veins.

Figure 2.—Encapsulated adenoma in 14-year-old female who had never taken oral contraceptives or other medication. *Note* hematoma within unruptured tumor. A 6-cm ruler is alongside.

Figure 3.—Gross appearance of classical FNH without hemorrhage or necrosis.

GUEST EDITORIAL by Fred Rapp

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Question: Do Herpesviruses Cause Cancer? Answer: Of Course They Do![1]

FOR 65 YEARS virologists and their colleagues in allied sciences have tried to show for humans what Ellermann and Bang (*1*) and Rous (*2*) demonstrated for the chicken in the first decade of the twentieth century. This long, costly, and frustrating search for a human cancer virus similar to the RNA-containing C-type particles isolated from the chicken, from a variety of rodents, and more recently from subhuman primates, has yielded minimal results in erms of isolates that might be etiologic agents in human neoplasm. Even the 2 most recent nominees, the ESP-1 (*3*) and RD-114 (*4*) viruses, are clouded by the methods used to isolate them and seem more likely to be murine and feline than human viruses.

The overwhelming attack on this problem through study of RNA-containing tumor viruses and the popularity of a highly publicized theory (*5*, *6*) to explain how such viruses can cause cancer have been accompanied by excellent work at the molecular level which supports theories that such viruses can redirect cells. Thus, the important demonstration that these viruses are accompanied by an RNA-directed DNA polymerase or reverse transcriptase (*7*, *8*) has served as a scaffold for understanding how such viruses may cause the synthesis of nucleic acids able to integrate into the host genome. The molecular probes that have been fashioned to detect such nucleic acids are now used to search for equivalent virus genomes in human cells, as discussed recently by Green and Raskas (*9*). However, such probes, although sensitive, are ultimately inappropriate for isolation, much less propagation, of human viruses that may be oncogenic.

Perhaps it is time to concentrate on viruses known to infect both man and animals and to be multipotential. Of particular interest would be those viruses with the ability to persist in the body for extended periods, coupled with the potential to cause a variety of clinical illnesses, one of which may be the neoplastic process. The viruses of one major group that has such qualities under natural conditions, both in animals and in man, are the herpesviruses.

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Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

Involvement of Herpesviruses in Animal Neoplasias

The early observations by Lucké (*10*) of the infectious nature of renal adenocarcinomas of frogs were followed some years later by demonstration through electron microscopy (*11*) that herpesvirus-like particles are often associated with such tumors (table 1). Similar virus-tumor associations are summarized in table 1. In addition to frog adenocarcinomas, it is known that herpesviruses can cause neoplasia in chickens (Marek's disease) (*12*), rabbits (*13*), guinea pigs (*14*, *15*), and a number of monkey species (*16–21*). A virus recently isolated from cattle (*22*) may fall into this category. Many such viruses have been isolated and shown to have oncogenic potential during the past few years. Many more viruses, especially as they are more carefully sought in subhuman primates, probably will be isolated and shown able to cause neoplasia when inoculated into an appropriate host. However, the disease has already been demonstrated to occur naturally (i.e., without inoculation of virus) in frogs, chickens, and possibly rabbits and guinea pigs and, therefore, appears to be transmitted from one animal to another. Thus it is not surprising, though significant, that an attenuated virus vaccine against Marek's disease of chickens can prevent the expression of the disease due to the wild-type virus found naturally in chickens (*23*).

Multipotential of Human Herpesviruses

All herpesviruses are able to cause an acute disease in man after the primary infection, which generally occurs early in life. These primary infections are characterized by cell destruction, are often systemic, but are frequently self limiting. Once the primary infection (which may be subclinical) has subsided, many herpesviruses can establish persistent or latent infection, often for the life of the host (*24*). These infections exist in the presence of circulating neutralizing antivirus antibody and in the absence of manifest clinical disease.

Perhaps the most interesting of these phenomena and certainly the best studied is the secondary and latent infection caused by herpes simplex virus (HSV). After the primary infection, infectious virus cannot be recovered until certain changes in the internal environment or nonspecific external stimuli induce virus production. This is generally followed by clinical illness, of which the fever blister is the best example. Experiments with organ cultures from mice and rabbits latently infected with HSV suggest that the trigeminal ganglia harbor the virus in a nonproductive infection between clinical episodes (*25*, *26*); in culture, virus can be recovered from the ganglia. Indeed, a recent report claims similar success in recovery of virus from human cases in which the trigeminal ganglion was co-cultivated with green monkey kidney (vero) cells (*27*).

Many stimuli for virus replication in vivo are accompanied by changes in temperature and it is, therefore, interesting that the herpesvirus implicated in the Lucké adenocarcinoma of frogs is replicated only when the frog or its tissues are maintained for a long time at low temperature; it is never found in frogs or in tumors of frogs during the summer (*28*, *29*). Survival of latent herpesviruses probably depends on the ability of the cells to resist virus replication, since synthesis of infectious herpes virions seems always accompanied by cell destruction (*30*, *31*). In the absence of an animal reservoir, the mechanism for survival of the human herpesvirus requires infection of the host in a primary, generally nonfatal, infection. The viruses

TABLE 1.—*Links between animal herpesviruses and cancer*

Year	Virus	Host	Observation
1938	Lucké	Frog	Renal adenocarcinoma.
1956	Lucké	Frog	Herpesvirus particles.
1967	Marek	Chicken	Virus involvement in Marek's disease.
1969	Marek	Chicken	Herpesvirus Marek's disease vaccine.
1969	*Herpesvirus saimiri*	Monkey	Lymphoma and acute lymphocytic leukemia.
1971	*H. sylvilagus*	Rabbit	Lymphoma.
1972	*H. ateles*	Monkey	Lymphoma.

can then spread to other hosts and can also establish nonproductive infections. The virus genome can subsequently be induced to replicate in the localized areas of involvement and this can lead to infection of other susceptible individuals. This seems true for varicella-zoster virus, as well as for HSV (*24*). This theory requires that these viruses and certain host cells form an intimate relationship for extended periods, during which the virus genome is preserved without damage to the host cell.

The Case for Epstein-Barr Virus

The reluctance of many scientists to accept the Epstein-Barr virus (EBV) as a probable human tumor virus is only slightly less than astonishing. The virus was first demonstrated (table 2) in a cell culture derived from Burkitt's lymphoma (*32*). Such cells, as well as cells of the tumor itself, are now known to contain virus-specific DNA (*33–35*). This virus DNA is associated with that of cellular DNA, though the molecular connection between the 2 nucleic acids remains unclear (*36*). Most cell lines derived from the Burkitt lymphoma can be induced to synthesize virus by exposure of the cells to halogenated pyrimidines (*37*, *38*). Clones of somatic cell hybrids derived by fusing Burkitt lymphoblastoid cells with other human cells also carry the virus genome which can be induced to replicate after exposure to 5-iododeoxyuridine (*39*). Exposure of normal human peripheral white cells or lymphoid cells of fetal origin to EBV-carrying cells or EBV itself gives the recipient cells ability for long-term culture (*40–44*), a characteristic not ordinarily found in the absence of a transforming virus. Sera from certain groups of cancer patients, including those with Burkitt lymphoma and nasopharyngeal carcinoma, almost always contain antibodies to one or more components of EBV (*45*). It is also fairly well established that this virus may represent the etiologic agent of infectious mononucleosis (*46*). The bothersome question of where this virus multiplies in man may be near the point of resolution, because a number of investigators have shown recently that transforming viruses resembling EBV can be isolated from the upper respiratory tract during acute stages of infectious mononucleosis (*47–49*).

TABLE 2.—*Links between human herpesviruses and cancer*

Year	Virus	Observation
1964	EBV	Virus in cultured cells from Burkitt lymphoma.
1966	EBV	Antigenicity of Burkitt cells: serology and epidemiology.
1968	EBV	Importance of EBV in establishing continuous human lymphoid cell lines.
1969	HSV-2	Epidemiology of cervical carcinoma.
1970	EBV	Virus DNA in Burkitt cells.
1971	HSV-1	In vitro transformation of mouse cells.
1971	HSV-2	In vitro transformation of hamster cells.
1972	EBV	Association of virus and cell DNA.
1972	HSV-2	In vitro transformation of human cells.

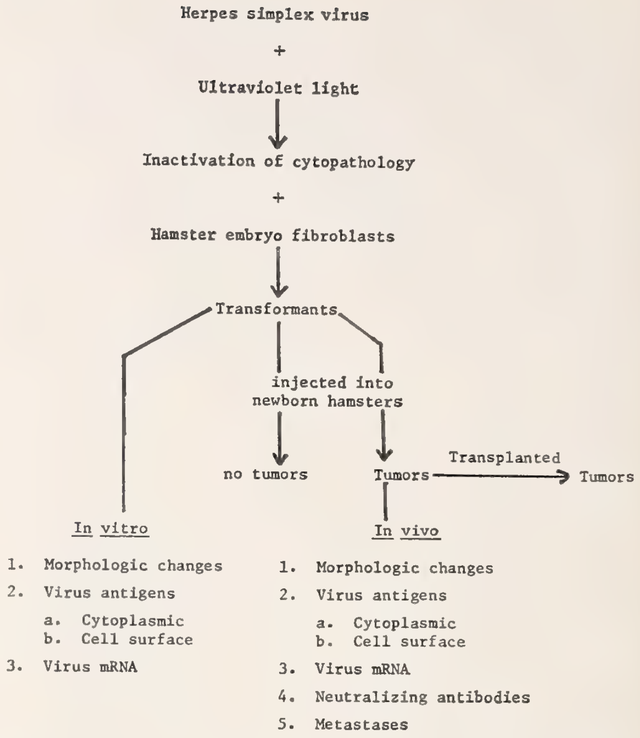
EBV seems to meet all the criteria of a tumor virus. The genetic information of the virus is regularly found in certain types of tumors and is associated with the host genome. The virus can convert normal cells into cells with infinite replicative capabilities; cells harboring the virus genome are often altered at the level of the cell membrane (*45*). EBV is widespread in the population; it has the ability to cause clinical infection with at least one recognizable (and non-neoplastic) entity (infectious mononucleosis). It is difficult to conceive of experiments that would yield further meaningful data, applicable to the human population, on the function of this virus in human neoplasia. This is, of course, a problem which will be encountered with all human isolates when we seek evidence that conclusively links a type of virus and a neoplastic disease.

The Case for Herpes Simplex Virus

Investigations of herpes simplex virus in human cervical carcinoma (table 2) were begun when it was observed that this virus could be divided into at least 2 groups (*50*), now known as herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). Although these viruses were originally separated by serologic techniques, nucleic acid hybridization studies with a number of virus isolates have confirmed that they differ markedly in nucleotide sequences (*51*, *52*) and consequently differ in a number of biologic properties (*53*). For this discussion, an important fact is that most strains of type 2 virus have been isolated from the genital tract, whereas viruses isolated from other sites tend to be type 1. Both types cause latent infections. Centifanto and colleagues (*54*) claim that the male genitourinary tract harbors the virus; the female may be infected during sexual intercourse. Early studies demonstrated a higher incidence of antibodies to HSV-2 in women with cervical carcinoma when compared to control groups (*55–57*). Although analyses of data collected in various parts of the world have yielded varied conclusions (*56–60*), there is general agreement that the virus infection and the disease may be associated. Although they have not been found in cervical tumors (*61*), virus antigens have been detected in exfoliated anaplastic cells (*62*) from patients with cervical carcinoma. Unfortunately, some of these results have not been confirmed and may be due to latent infection of cells in the genital tract.

Early experiments designed to determine whether this virus is oncogenic generally involved inoculating the agent into newborn animals. These experiments often failed because of the virulence of this virus, though a few tumors were observed (*63*) in which neither virus antigens nor other virus markers were detected. A direct attack on the possibility that this virus has oncogenic potential was carried out using irradiated virus to transform cells in vitro (text-fig. 1). The rationale used was that radiation of known tumor viruses reduces infectivity at a faster rate than transforming activity (*64–66*); it was hoped that the procedure might destroy the ability of the virus to cause cytopathology without destroying the potential to transform cells in culture.

Independent groups have now demonstrated the feasibility of this approach (tables 2, 3). Munyon and colleagues (*67*) transformed the mouse L cell lacking the ability to synthesize the enzyme thymidine kinase (LTK⁻ cells) to the thymidine kinase positive phenotype with irradiated HSV-1. The same procedure has been successful with HSV-2 and the mouse L cells (*68*). Duff and Rapp (*69*, *70*) transformed hamster cells in vitro with irradiated HSV-2 (text-fig. 1); the morphologic transformants that arose were highly oncogenic, carried virus antigens in some cells, and could synthesize virus-specific mRNA (*71*, *72*). Many isolates have been tested thus far, and several HSV-2 serotypes have been able to transform hamster cells in vitro though the cells are not invariably oncogenic (*53*). At least two HSV-1 isolates have yielded similar results (tables 3, 4).


Herpes simplex virus
+
Ultraviolet light
Inactivation of cytopathology
+
Hamster embryo fibroblasts
Transformants
injected into newborn hamsters
no tumors
Tumors
Transplanted
Tumors
In vitro
1. Morphologic changes
2. Virus antigens
a. Cytoplasmic
b. Cell surface
3. Virus mRNA
In vivo
1. Morphologic changes
2. Virus antigens
a. Cytoplasmic
b. Cell surface
3. Virus mRNA
4. Neutralizing antibodies
5. Metastases

Text-figure 1.—Summary of protocol employed to demonstrate transformation of hamster embryo fibroblasts in vitro by herpes simplex viruses.

No virus has been recovered from the transformed cells, though herpesvirus-like particles are sometimes observed in degenerating cells (*73*). No evidence for synthesis of the group-specific antigens of hamster leukemia viruses or C-type particles has been found (*74*).

An independent series of experiments on a stable line of hamster cells and irradiated HSV-2 has also resulted in transformants that synthesize virus antigens; the originally weakly oncogenic cell line became strongly oncogenic after transformation (*75*). However, that many HSV isolates do not transform cells in culture (table 4) suggests either that more sensitive techniques will be required or that these viruses differ in this ability. If the latter is true, it will be extremely important to determine other properties in which they differ that may concern their transformation capabilities.

TABLE 3.—*Evidence for transformation by herpes simplex viruses*

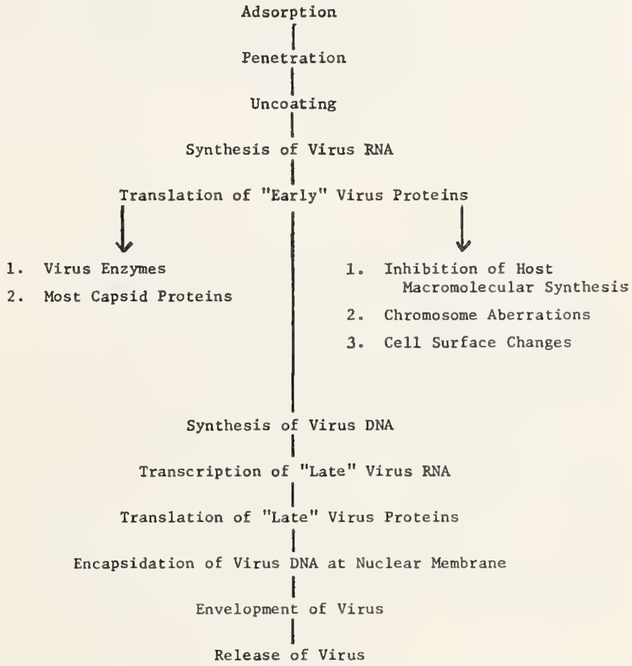
Year	Virus type	Treatment of viruses	Cell type	Criteria
1971	1	Ultraviolet irradiation	Mouse L cell line	Thymidine kinase.
1971	2	Ultraviolet irradiation	Hamster embryo fibroblasts.	Morphology, antigenicity, immortality, oncogenicity.
1972	1	Ultraviolet irradiation	Hamster embryo fibroblasts.	Morphology, antigenicity, immortality, oncogenicity.
1972	2	Ultraviolet irradiation	Mouse L cell line	Thymidine kinase.
1972	2	Cells and virus at 42° C	Human embryo lung fibroblasts	Morphology, antigenicity, immortality.
1972	2	Ultraviolet irradiation	Hamster cell line	Morphology, antigenicity, oncogenicity.

TABLE 4.—*Transformation of hamster embryo fibroblasts by herpes simplex viruses*

HSV type	Number of strains tested	Number of strains positive
1	12	2
2	15	7

Darai and Munk (*76*) recently reported the transformation of human embryonic lung cells by *nonirradiated* HSV-2. These investigators infected the cells and maintained them for extended periods at high temperature (42° C). Normal cells were destroyed, but transformed cells survived to grow into established cell cultures. These cultures have a life time longer than the normal diploid cells. It will be interesting to determine their antigenic makeup and the possible association of virus DNA with the host genome.

The increased emphasis on research with the herpesviruses is yielding new clues about replication of these viruses (text-fig. 2). It is generally considered that cells productively infected experience chromosome aberrations (*77–80*) and are invariably destroyed after early inhibition of host macromolecular synthesis (*30*, *31*). These observations are based on studies done in cell culture and it appears logical, in light of what is known about these viruses under natural conditions, that some cells can harbor the virus or the virus genome without being destroyed. More emphasis on this type of virus-cell interaction is obviously needed. Thus use of virus mutants for genetic studies under a variety of conditions in various host cells should yield information about the relationships that these viruses can establish with host cells.


Adsorption
Penetration
Uncoating
Synthesis of Virus RNA
Translation of "Early" Virus Proteins
1. Virus Enzymes
2. Most Capsid Proteins
1. Inhibition of Host Macromolecular Synthesis
2. Chromosome Aberrations
3. Cell Surface Changes
Synthesis of Virus DNA
Transcription of "Late" Virus RNA
Translation of "Late" Virus Proteins
Encapsidation of Virus DNA at Nuclear Membrane
Envelopment of Virus
Release of Virus

TEXT-FIGURE 2.—Steps in the replication of herpes simplex viruses in permissive cells.

Also, many cells may contain a repressor of virus replication that lets the virus and the cell remain in equilibrium without damage to either virus or host genome. Evidence of such a repressor is already available; extracts from cells transformed by the virus can reduce or prevent plaque formation (in essence, cytopathology) when recipient cells are exposed to the extract before the introduction of infectious virus (*81*). The mechanism of repressor action is unknown but may well be at the transcription level, since early virus transcription apparently drastically affects host macromolecular synthesis. As it is also known that most virus-specified events in a productive infection take place in the absence of virus progeny DNA synthesis, the only reasonable pathway for cell survival would be an early block in protein synthesis specified by the virus. This early block would prevent expression of most virus functions, and the presence of the virus in the cell would therefore be extremely difficult to detect under those conditions, as neither virus antigens nor large quantities of virus-specific nucleic acids would be produced. Recent experiments in which antimetabolites have induced expression of EBV antigens and virus (*37–39*) offer some hope that similar technology might reveal other situations in which the virus genome is harbored by its host cell. Alternatively, HSV will possibly transform cells only when a portion of its genome is involved; this transformation by a defective genome might also be accompanied by repression but it would theoretically not be possible to induce such cells to synthesize infectious virions.

Future Expectations

Additional herpesviruses will probably be isolated from various species and these viruses may have oncogenic potential in appropriate systems. Emphasis will clearly be on viruses from subhuman primates. Also, more emphasis should be placed on other herpesviruses, such as varicella and cytomegaloviruses, that are in the human body for extended periods. Technology to demonstrate transforming potential of these viruses must be improved to enable greater sensitivity. This should be possible as the properties of these viruses are more carefully explored and as more focus is placed on the role of these properties in oncogenesis.

The warning of the EBV system forces the conclusion that it will be extremely difficult to demonstrate directly the function of these viruses in human neoplasia. The more virulent the virus, the less likely that virus-specific products will be easily detected in the transformed cells. Thus continued accumulation of circumstantial evidence will be necessary to link these viruses to the etiology of the various neoplasms with which they have already been associated. It may even be predicted that transformed cells will contain <1 complete virus genome per cell, especially if a defective virus genome converted the cell. The day in court for herpesviruses will come when the evidence is substantial enough to suggest to most reasonable scientists that these viruses qualify for the role of oncogenic agents. Whether the conversion of normal cells into tumor cells is a direct property of the viruses or whether these viruses simply act in concert with other agents, such as other viruses or chemicals, to effect the neoplastic process will require careful investigation. For now, we know that the herpesviruses are often at the right place at the right time, and they appear to have the properties that qualify them as candidates for the role of human cancer viruses.

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ADDENDUM

Experimental demonstrations that a variety of human herpesviruses transform normal cells to a malignant phenotype have not definitively established that all such viruses can effect oncogenic transformation. Human cytomegalovirus can establish persistent infection of human cells leading to the selection of transformed cells that produce tumors when transplanted to nude mice (*1*). All cells transformed with HSV or cytomegalovirus contain virus-specific antigens and nucleic acids. It appears that only a portion of the virus DNA is required for cell transformation. Although the amount differs depending on the investigation, between 5 and 40% of the virus genome is present in rodent cells transformed by HSV.

In addition to African Burkitt's lymphoma, EBV has now been linked with nasopharyngeal carcinoma of the Southern Chinese population. Both neoplasias have EBV DNA associated with the tumor cells and, though rarely expressed, the virus can be induced to replicate by cocultivation procedures. Associated with that expression is an intranuclear antigen (EBNA), which resembles the T-antigens of papovaviruses and adenoviruses. The role of this antigen is unknown but it apparently is associated with chromosomes.

The extensive search for nucleic acids in cervical tumors by in situ hybridization (*2*) has now revealed that virus nucleic acids are present in most of the tumors but not in neighboring normal cells. These results foster the belief that HSV is associated more frequently with cervical tumor cells than previously determined and that this phenomenon may resemble the observed relationship of EBV to Burkitt's lymphoma and nasopharnygeal carcinoma. Moreover, in a recent study, Huang and Roche (*3*) detected cytomegalovirus nucleic acids in certain carcinomas of the colon.

Thus the association of human herpesviruses with various human neoplastic diseases is quantitatively stronger than for any other virus group, with the possible exception of hepatocarcinoma which has been linked to chronic hepatitis-B. Molecular probes generated by restriction enzymes and cleavage of virus DNA should reveal further virus associations and lead to a type of "molecular epidemiology," which may help resolve the question of etiology of human neoplasias. In addition, increasing venereal spread of HSV type 2 may result in larger numbers of cervical neoplasias in future years; this occurrence would solidify the association of HSV with the disease.

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GUEST EDITORIAL **by M. A. Epstein**

JNCI 56:697–700, 1976

Epstein–Barr Virus—Is It Time To Develop a Vaccine Program?

Whatever may be thought of the magnitude of effort and expense devoted to research on tumor viruses or the various directions in which particular thrusts are encouraged from time to time, there can be no controversy over the ultimate goal. Quite simply, the goal must be to acquire sufficient knowledge of viral oncology ultimately to eliminate virus-induced malignant tumors of man.

The main steps toward this objective were clearly established when the Special Virus–Leukemia Program (which later became the Special Virus–Cancer Program) was initiated in 1964. Viral agents were to be sought in tumors and their constant association with human cancer was to be established. Efforts were to be made to determine whether at least one human cancer had such an associated virus as cause, and if so, an effective antivirus vaccine was to be developed for the control of the tumor (*1, 2*).

In recent years, several suspected human tumor viruses have been identified; some have survived scrutiny and are currently under investigation. But with any such agent, direct proof of a role in the cause of human malignancy is likely to be impossible to obtain without violating ethical barriers, and only indirect and circumstantial evidence will be available. The question will, therefore, always remain as to the value of accumulating yet more and more information on the association of an agent with a given human tumor, since this will never provide a final, definitive answer. At some stage a more dynamic approach must be devised, and it may well be that this should be undertaken soon.

Of the viruses currently suspected of involvement in the etiology of human cancer, it is not unreasonable to say that the Epstein–Barr virus (EBV), discovered in relation to Burkitt's lymphoma (*3*), is the only really convincing candidate at present. The reasons are that EBV fulfills some of the major requirements envisaged at the start of the Special Virus–Leukemia Program: It is an unusual new human virus, it shows a constant close association with two specific types of human malignant tumors, and its biologic behavior includes features not unlike those of known oncogenic animal viruses. Thus properly authenticated cases of African Burkitt's lymphoma and nasopharyngeal carcinoma (NPC) only occur in individuals infected by the virus; the viral DNA is present in the tumor cells and determines the expression in them of virus-coded neoantigens. Therefore, it is not surprising that virus production can be activated in some of the tumor cells by various laboratory procedures. The virus is a powerful stimulator of lymphoproliferation in vivo as the cause of Paul–Bunnell-positive infectious mononucleosis (IM); it is a powerful stimulator of lymphoproliferation in vitro, conferring the property of continuous growth on normal human B lymphocytes with many changes analogous to malignant transformation; and it is carcinogenic experimentally in vivo, causing malignant lymphoma in inoculated South American subhuman primates, with Koch's postulates fulfilled in the system. Animal herpesviruses behaving similarly to EBV cause malignant lymphoma or carcinoma in natural or experimental hosts. Recent publications have documented each of these points (*4–8*), but, as expected, this impressive evidence suggesting that EBV plays some part in the etiology of African Burkitt's lymphoma and NPC (with whatever cofactors may be involved) is wholly circumstantial and inferential.

However, it appears that presently we know more than enough to incriminate EBV as a prime suspect for the role of human tumor virus, and the question of direct proof must now seriously be considered. As has already been suggested (*9*), the only way to resolve this difficulty would be the development of an experimental vaccine to be used not only with the long-term hope of controlling human malignancies, but in the short term as a means of establishing the carcinogenicity of EBV.

The difficulties in preparing and administering to human populations a safe and effective vaccine for a suspected tumor virus are obvious, but recent work indicates that much less may be involved than has sometimes been feared in the past. There is also the question whether the considerable efforts that such a program would need are worthwhile. Apart from the scientific objective of showing that at least one human tumor has a viral etiology, there are also important practical reasons for perservering with EBV in this respect. Although Burkitt's lymphoma occurs frequently in limited areas and does not involve particularly large numbers even in these areas (*10*), an effective vaccine to EBV protecting against Burkitt's lymphoma would demonstrate some causative role for the agent in this tumor and thus would immensely strengthen the likelihood that EBV is likewise an etiologic agent in NPC. A high incidence of NPC is also limited in its geographic distribution, but here the limitation is racial, being confined to Southern Chinese people, and there are very large numbers of these in various parts of the world. Thus although Burkitt's lymphoma may be a minor medical problem, NPC is considerably important in terms of world health, since it is the commonest cancer of adult males and the second commonest cancer of adult females of Southern Chinese

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

origin (*11*) who, with related races, form about one-third of the population of the world (*12*).

Any scheme for an anti-EBV vaccine will need to be performed in two steps: 1) a small-scale pilot experiment to prove the carcinogenicity of the virus in relation to African Burkitt's lymphoma; and 2) a more complicated, long-term project, the wide-scale vaccine control, not only of Burkitt's lymphoma, but more importantly of NPC with its great significance in terms of numbers involved. There are special advantages afforded by the EBV and African Burkitt's lymphoma system; as pointed out elsewhere (*4*), since EBV causes IM, the antivirus efficacy of any vaccine could be tested by its ability to protect those at risk from natural primary EBV infection accompanied by this disease. In addition, there are well-recognized areas of high endemicity of African Burkitt's lymphoma, where the effect of a vaccine on tumor incidence could be tested. Finally, since African Burkitt's lymphoma is a disease with a peak incidence in children around the age of 6, it should be possible to vaccinate all 0- to 1-year-old members of a population in an endemic area and to judge the effect on tumor development within 5–10 years; thus an answer would be obtained far more rapidly than possible with most other human tumors which occur mainly late in life.

Concerning the practicality of vaccination, control of a naturally occurring, herpesvirus-induced malignant tumor by an antivirus vaccine was demonstrated 6 years ago when live, apathogenic herpesvirus vaccines were introduced to protect chickens against the development of Marek's lymphomas (*13, 14*). However, the applicability of a live virus vaccine to the human situation is obviously doubtful because it is impossible to administer a suspected tumor-inducing virus, however attenuated, to man. Even a conventionally inactivated virus of this kind would raise strong objections for use as a human vaccine because of the problem of proving total inactivation and the possibility that traces of viral nucleic acid in such a preparation could induce malignant transformation. However, further progress with vaccines against oncogenic, animal herpesviruses has indicated the ways in which such difficulties can be overcome. It is now known that chickens are significantly protected against lymphomas of Marek's disease by vaccines free of viral nucleic acid. Lesnick and Ross (*15*) reported success with a vaccine consisting only of soluble viral antigens extracted from Marek's virus-infected tissue culture cells by treatment with nonionic detergent; other workers (*16*) have used highly purified plasma membranes from similar virus-infected cells as a vaccine and have thereby reduced the mortality rates from Marek's lymphomas by 94% when the vaccinated chickens were subsequently challenged with virulent virus. The whole question was recently moved closer to man when Laufs and Steinke (*17*) showed that subhuman primates can also be successfully protected against a herpesvirus-induced malignant lymphoma. The researchers used heat and formaldehyde to inactivate *Herpesvirus saimiri,* which is otherwise carcinogenic in marmosets (*18*), and complement-fixation tests have shown that the inactivated vaccine maintains considerable, specific viral antigenicity after these treatments.

Numerous cottontop marmosets have been immunized with the vaccine, remained well, and developed high titers of neutralizing and complement-fixing antibodies to the virus. When last reported (*17*), 22 immunized animals had been challenged with a large tumor-inducing dose of virulent *H. saimiri* and continued alive and well for many months, whereas inoculated control animals died of malignant lymphoma within 52 days. There seems no reason to doubt that nucleic acid-free vaccines will soon be developed for *H. saimiri* like those already used against the herpesvirus causing Marek's lymphomas in chickens. Development of a viral, nucleic acid-free vaccine for EBV along similar lines will clearly meet many of the previous ethical objections to the concept of a vaccine for this suspected human tumor virus, particularly if purified antigens are used.

The methodology for preparation of nucleic acid-free, herpesvirus vaccines to control animal tumors is rapidly progressing, and the application of the necessary techniques to EBV is unlikely to present significant difficulties. It has sometimes been argued that the absence of a fully permissive cell for the replication of EBV presents an insuperable problem for vaccine production, but this argument misses the whole point; with this system, virus production is both unnecessary and undesirable. There are available appropriate, continuous human lymphoid cell lines that do not replicate EBV but do have virus-determined membrane antigens expressed on the cell surface, and it seems that antibodies to these membrane antigens also have virus-neutralizing activity (*19–22*). No difficulty or special cost is involved in growing such cells on a large scale, and techniques both for preparing cell membranes from human lymphoid cells and for purifying antigens from the isolated membranes are well tried (*23–26*).

Two main safety factors are of fundamental importance in the administration of such a vaccine to man. First, the total absence of viral DNA must be confirmed in the material to be inoculated; this is probably much less difficult than the more complicated problem presented by the safety testing of polio vaccines 20 years ago. Second, and perhaps more important, safety also demands demonstration that the vaccine does not engender immunopathologic complications after administration. Although this hazard seems unlikely if purified membrane antigens are used, the problem of enhancement must be considered. It could be argued that use of a killed vaccine for immunization of a population at risk for the development of Burkitt's lymphoma would be justified only if immunity were maintained by booster doses throughout life, since a fall in immunity might permit EBV infection with the development of blocking factors capable of enhancing the growth of any malignantly transformed Burkitt's lymphoma cells that subsequently emerged. However, it could equally be that a high secondary antibody response to infection may result from previous but lapsed vaccination, may directly limit the growth of such tumor cells, and may thus be beneficial. In any event, these questions can now be tested experimentally with the availability of both subhuman primates susceptible to tumor induction by EBV (*7, 8*) and of the *H. saimiri* model with the use of killed vaccine in marmosets (*17, 18*).

Susceptible subhuman primates are also necessary for the essential in vivo laboratory testing of the protective efficacy of a vaccine. Once protection has been demonstrated satisfactorily in animals and the safety problems have been resolved, efficacy of the vaccine in preventing EBV infection in man is the next step; this can be investigated in the context of EBV seronegative young people at risk for primary infection in the age group in which the primary infection is accompanied by a 50% or higher incidence of clinical IM (*27*).

If we assume that the preparation of a membrane vaccine, the safety testing of such a vaccine, the authentication of its effectiveness in animals, and the prevention of IM in EBV-negative individuals at risk can all be successfully achieved, the vaccine could then be considered for testing in a field trial to show that prevention of EBV infection significantly reduces the occurrence of Burkitt's lymphoma in a high-incidence area. It is obvious that EBV cannot be solely responsible for the induction of Burkitt's lymphoma in endemic areas and that some climate-dependent cofactor such as holoendemic malaria (*28*) must also pay a part; however, if EBV is a necessary component, then prevention of infection by a vaccine must have an effect on the subsequent incidence of the tumor in the trial area. This would parallel the situation with the herpesvirus of Marek's disease, in which the agent is widespread in chicken populations but exerts its oncogenic potential only in combination with certain individual, environmental, and genetic circumstances (*29*), and in which protection against virulent virus prevents development of lymphomas.

For a field trial on children in an area where Burkitt's lymphoma is highly endemic, it is doubtful whether a membrane vaccine could induce sufficiently long-lasting immunity, and revaccination would be required to maintain protection from the earliest postnatal months to the age of 5 or 6 years, when African Burkitt's lymphoma has its peak incidence. However, even in remote and undeveloped regions, it is perfectly possible to successfully mount complicated programs involving considerable numbers. The West Nile District of Uganda Project currently functioning under the auspices of the International Agency for Research on Cancer (*30*) is a Burkitt's lymphoma-related example of such a program that depends on relatively simple financial and logistic support. Blanket vaccination of an appropriate postnatal age cohort in an area such as the West Nile District is not likely to prove any more complicated even with the necessity for subsequent booster doses. The relatively small West Nile District has a dense population with a sufficiently high tumor incidence (*31*) for a drop referable to an antecedent vaccination program to be recognizable. It would thus be an appropriate place to test such a vaccination scheme, as would many other places in which similar high incidences of Burkitt's lymphoma are also known.

Should the results of such a vaccine program indicate that EBV is causally related to African Burkitt's lymphoma even in association with cofactors, the case will be immensely strengthened for its consideration as a likely etiologic agent in the induction of NPC. Vaccination to protect against this malignant tumor of later life poses considerable problems in the maintenance of immunity to EBV over many years, and modifications would be required in the type of vaccine to be used. The difficulties here should not be minimized, but since NPC is the commonest tumor among a substantial section of the world population, no effort should be spared if control seems possible. If such control of an exceptionally common tumor among Western populations were in sight, support would be forthcoming; the effort should surely be similarly worth consideration for another large and equally significant area of the world. If the recent developments and possibilities outlined above are considered, it does not seem unrealistic to suggest that planning for the investigation of the many problems ahead would not be premature at the present time.

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ADDENDUM

Since the ideas in this Guest Editorial were first brought together, they have continued to be highly relevant to the central goal of tumor virology, i.e., the prevention of a human cancer by the vaccine control of an oncogenic virus. Indeed, the appropriateness of making a start on such a policy has increased considerably in the light of recent findings on EBV in relation to Burkitt's lymphoma.

In addition to the well-known facts that suggest an oncogenic role for the virus in this disease that were set out in the Editorial, it is now clear that patients with tumors show a special pattern of antibodies to the viral antigens and that these vary with disease events in a remarkably specific way (*1*). Furthermore, an important prospective study on 42,000 children in an area of high tumor incidence by the International Agency for Research on Cancer has just established that those children destined to develop endemic Burkitt's lymphoma have all been infected by the virus for long periods and have shown a significant difference in their response to it compared with controls (*2*). The data on this unusual response permitted the calculation of the risk factor attached to it and have shown it to be about 30 times higher than that for the control population (*2*). Such a risk factor is indeed striking and appreciably greater even than the one generally accepted as establishing an etiologic relationship between heavy cigarette smoking and carcinoma of the bronchus.

The fact that the virus behaves so similarly to known oncogenic animal viruses (*3*), taken in conjunction with the newly demonstrated risk factor, seems clearly to provide all the possible circumstantial evidence that can reasonably be required to establish EBV as the cause of this human cancer (*4*). The need to obtain direct proof of this by the methods urged in the Editorial as a preliminary to attempting the difficult long-term vaccine control of the numerically more important EBV-associated nasopharyngeal carcinoma is thus both greater than before and more compelling.

At the same time, progress has been made in the methodology required for such work. New and simpler methods for obtaining cell membranes carrying herpesvirus antigens have been reported (*5*), have yielded antigenic preparations (*6*), and will doubtless now be used to protect subhuman primates against *Herpesvirus saimiri*-induced malignant tumors; improvements are also taking place in techniques for the enhancement of antigenicity.

The time was ripe in 1976 to consider an EBV vaccine program; it is certainly time now to implement it.

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GUEST EDITORIAL

Allen W. Cheever, *M.D.*[1]

JNCI 61:13–18, 1978

Schistosomiasis and Neoplasia

Napoleon's troops are said to have referred to Egypt as the "land of menstruating males." Hematuria from schistosomal cystitis was so common that the passage of blood in the urine was taken as an indication of puberty. Schistosomes are parasitic flatworms that are transmitted through freshwater snails. Adult worms of two of the three species commonly infecting man (*Schistosoma mansoni* and *S. japonicum*) live in mesenteric venules and produce pathologic changes mainly in the liver and colon. The third species, *S. haematobium*, resides mainly in venules of the bladder and ureters. The living adult worms provoke little reaction, and most pathologic changes are caused by host inflammatory reaction to eggs laid by the worms. Eggs retained in the tissues slowly release antigen into the surrounding tissues (*1*). However, the eggs live for only a few weeks, during which time the principal reaction to them takes place. The fate of dead eggs varies greatly depending on the species of both the worm and its mammalian host. In man, eggs of *S. mansoni* are rapidly cleared from the tissues, whereas eggs of *S. haematobium* tend to calcify and persist for prolonged periods (*2*). The pathogenetic significance of calcified eggs is not defined, but these eggs are probably inert. In endemic areas, schistosome infections are usually acquired at an early age and persist for many years. Although they continue to lay eggs throughout the infection, the worms do not multiply within the body. Infections may thus be light or heavy, and the pathologic changes vary accordingly. Under most epidemiologic circumstances, infection intensity in man peaks between the ages of 15 and 20 years. Immunity to superinfection is readily demonstrated in animals but has not been well documented in man (*3, 4*).

SCHISTOSOMIASIS AND CANCER OF THE URINARY BLADDER

Does Schistosomiasis Cause Bladder Cancer?

The frequent association of *S. haematobium* infection with bladder cancer, the high prevalence of bladder cancer in some areas endemic for schistosomiasis, and the distinctive morphology of the bladder tumors led investigators at the turn of this century to postulate a causal association between schistosomiasis and bladder cancer in Egypt (*5, 6*). In contrast to other bladder cancers, those associated with schistomiasis are usually squamous cell in type, seldom originate in the trigone, and occur in relatively young individuals (*7–9*). A series of 229 cases recently reported from Egypt by El Boulkany et al. (*10*) is typical. Two-thirds of the tumors were squamous cell, less than 6% originated in the trigone, and the mean age of the patient at the time of diagnosis of the cancer was 47 years. Khafagy et al. (*11*) found multiple infiltrative primary tumors in 22% of 86 infected cases and carcinoma in situ in 41%. Ishak et al. (*7*) noted squamous cell carcinoma in situ in 31% of 49 bladders containing squamous cell carcinomas. Squamous metaplasia is common and occurs most frequently in bladders bearing squamous cell carcinomas. Cystitis cystica, cystitis glandularis, and colon metaplasia are also frequent (*7*). Rigorous statistical proof of the association between bladder cancer and *S. haematobium* infection has not been obtained, though most published data tend to confirm the association (*12–14*). In areas where *S. haematobium* infections are less prevalent and less intense than in Egypt, most authors note an association of squamous cell bladder cancer with schistosomiasis [(*15–22*) are the more recent publications], but conflicting reports also exist (*23*). In some localities in Africa other identifiable factors clearly are more important than schistosomiasis—for example, urethral stricture in Uganda is frequently associated with squamous cell carcinoma of the bladder, but these tumors most commonly arise in the bladder trigone (*24*).

The apparently overwhelming yet unproved association of schistosomiasis and bladder cancer has perhaps discouraged further studies of the problem. No epidemiologically oriented study of the association has been undertaken since the report of Mustacchi and Shimkin published 20 years ago (*12*). Thus, although our knowledge of the morphology and behavior of the tumors has progressed (*7, 10, 11, 25, 26*), our knowledge of the causal connection has not. A second potent deterrent to clarification of the problem is the great difficulty one may encounter in planning and executing a proper study. For example, common sense would lead one to expect bladder cancer to be more frequent in heavily infected patients. The lower prevalence of schistosome-associated bladder cancer in countries with less severe schistosomiasis suggests the same conclusion,

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Abbreviation used: HbsAg=hepatitis B surface antigen.

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

as does the association of bladder cancer and occupational exposure to schistosome infection (*9*). However, meaningful determination of the intensity of infection is extremely difficult. The number of schistosome eggs passed in the urine probably reflects the intensity of infection (*2*), but, as previously noted, the mean age of infected individuals at the time of maximum egg passage is about 15 years. By the time cancer has developed, the schistosome infection is often inactive or minimally active. Masses of calcified eggs in the bladder often attest to the previous presence of heavy infection, though calcified eggs apparently persist in some individuals (*2, 27*) but not in others (*27*). Generally such calcified eggs are not passed in the urine where they could be detected or counted (*28*). For the pathologist to count the number of eggs in bladders removed for cancer is a simple matter, but to obtain similar data from a comparable population without bladder cancer is nearly impossible.

Two approaches to the study of infection intensity in patients with bladder cancer seem feasible. First, the "calcified bladder wall" in *S. haematobium* infection is not itself calcified but merely packed with calcified schistosome eggs (*29*). The shadow on the radiograph is related to the number of schistosome eggs in such bladders (*28*). The determination of the prevalence of cancer in groups with different infection intensity, judged from plain radiographs of the abdomen, is possible. Second, the transmission of schistosomiasis is often very focal, and to study otherwise similar groups of people in areas of high and low transmission is feasible when transmission is judged either from egg passage in younger patients or bladder calcification in older patients.

How Important Is Schistosomal Bladder Cancer?

No data exist that would allow one to reasonably estimate the morbidity and mortality caused by schistosomal bladder cancer. In two recent series of cases from Egypt (*30, 31*), about 2% of *S. haematobium*-infected patients seen at autopsy had bladder cancer. If all bladder cancers in infected patients are assumed to be fatal and to be caused by schistosomiasis, they would account for 16% of deaths related to *S. haematobium* infection in one series (*30*) and for 26% of deaths attributed to either *S. haematobium* or *S. mansoni* in the other (*31*). Twenty years earlier in the same hospital, bladder cancer was found in about 4% of infected cases (*14*). Much less sensitive techniques for the detection of schistosome infections were used in this earlier study. These data are from an urban university teaching hospital, and I have no idea what relation they may have to the mortality caused by schistosomal bladder cancer in the population at risk. Looked at from another point of view, bladder cancer is among the most frequent malignant neoplasms occurring in Egypt and Mozambique (*8, 13, 32*). Mustacchi and Shimkin (*12*) estimated that the relative risk of bladder cancer for patients with schistosomiasis in Tanta, Egypt, was 2.1-2.2, but they emphasized the need for better epidemiologic surveys. Whatever the past and present risk of schistosomal bladder cancer, the risk possibly will increase if exposure to other bladder carcinogens becomes more frequent.

What Are the Etiologic Factors in Schistosomal Bladder Cancer?

Chronic inflammation of the bladder and urinary retention were early noted as possible mechanisms by which schistosomiasis might predispose to bladder cancer. Inflammatory infiltrates and ulceration (usually microscopic) might decrease the effectiveness of the mucosal barrier to reabsorption of carcinogens in the urine (*33*). Urinary retention might also lead to increased absorption of carcinogens from the urine. A small amount of residual urine is frequently found in *S. haematobium* infection (*27*). Severe obstruction of the bladder neck is also described (*34*), though it is apparently infrequent (*31*). Elevated levels of urinary β-glucuronidase have been noted in *S. haematobium*-infected patients, and this enzyme may cleave glucuronides to yield carcinogenic products (*35, 36*). The urinary concentration of tryptophan metabolites, some known bladder carcinogens, was found to be increased in patients with *S. haematobium* infection (*37, 38*).

The possible presence of carcinogenic substances in the schistosome eggs has been little studied and has not been examined with more sensitive in vitro techniques such as cell transformation or mutagenic effects on bacteria. The effects of schistosome infection on the immune system might also affect carcinogenesis. The infections are prolonged with continued release of eggs and metabolic products into the circulation. Thus a major immune stimulus obviously exists. Immunodepression also exists but is generally specific, i.e., confined to reactions to the parasite and its eggs, but more generalized immunodepression has been described in some experimental situations. These findings and host immunity and hypersensitivity in general have been thoroughly reviewed by Phillips and Colley (*4*).

The interaction of bacteria in the urine with excreted chemicals must also be considered, e.g., the formation of nitrosamines from nitrites (*39*). Bacterial infection of the urine may be common in hospitalized patients with schistosomiasis, perhaps because of instrumentation of the urinary tract or because patients with bacteriuria are more likely to seek medical attention. However, with one exception (Laughlin L, Higashi G, Edman D, et al: Unpublished data), field studies have not shown increased bacteriuria in those individuals passing schistosome eggs in the urine (*40–42*). *Salmonella* bacteriuria is clearly more frequent in patients with *S. haematobium* infection than in those without (*43*), but again the prevalence of *Salmonella* bacteriuria outside of hospitals is low (*44, 45*). The low prevalence of bacteriuria in schistosome-infected patients does not rule out a role for bacteria in the pathogenesis of bladder cancer. One should also note that the field studies that have failed to link bacteriuria to egg excretion missed most patients with inactive schistosomiasis, and these individuals may have severe damage to the urinary tract as the result of previous schistosome infection (*30, 31*).

The clinical severity of *S. haematobium* infection varies greatly in different countries and in different areas within each country. Many differences appear to be caused by varied exposure to infection with resulting variation in intensity of infection. However, strains of *S. haematobium* from different areas also have distinctive behavior in both snails and experimentally infected mammalian hosts (*46*). The possible importance of the strain of schistosome for the production of bladder cancer is unknown, and genetically determined differences between patients who develop bladder carcinoma and those who do not have also not been investigated.

What Are Suitable Experimental Models of Schistosomal Bladder Cancer?

Experimental studies of schistosomal bladder cancer have been hampered by the lack of suitable experimental hosts. *S. haematobium* develops well in Syrian golden hamsters, in which bladder involvement is irregular. Other rodents tested have proved to be unsuitable hosts. Several nonhuman primate species are good hosts (*47*). Papillary and nodular transitional cell hyperplasia of the bladder can be produced with some regularity in capuchin monkeys. On the basis of the marked increase in thickness of the epithelium and the fusion of papillary growths, we believed these to be morphologically equivalent to grades I–II papillary bladder tumors of man. However, the tumor cells were well differentiated, and these lesions have not infiltrated the bladder wall or metastasized (*48, 49*). Several tumors, from which biopsy specimens were taken at cystotomy, have regressed, and in these animals the schistosome infection in the bladder had become inactive or minimally active (*49*). Increased levels of tryptophan metabolites in the urine of infected monkeys paralleled those found in infected persons (*50*). C-type virus particles were seen in a tumor from one monkey (*51*). Similar papillary and nodular epithelial hyperplasia was seen in talapoin monkeys (*48*), gibbons (*52*), and opossums (*53*). Talapoin monkeys and gibbons are not available commercially, and further experiments with opossums led to only irregular involvement of the bladder by the schistosome infection (*54*). In addition, spontaneous bladder tumors occur frequently in opossums.

A superficially infiltrative cancer of the bladder was produced in 1 cynomolgus monkey infected with a related schistosome species, *S. intercalatum* (*55*). However, this has proved not to be a reproducible phenomenon (Kuntz RE: Unpublished data). Cynomolgus monkeys are not appropriate hosts for *S. haematobium*, which produced only abortive infections with minimal production of eggs (Cheever AW, Duvall RH: Unpublished data). *S. haematobium* infection and *N*-methyl-*N*-nitrosourea apparently have additive effects on the hamster bladder, and similar studies have begun with baboons (*56*). Cocarcinogenic effects of *S. haematobium* and 2-acetylaminofluorene were reported for the mouse bladder by Hashem and Boutros (*57*), but the schistosome infection did not involve the bladder and the authors did not state whether the mice became infected by the schistosome larvae to which they were exposed.

Injection of dead schistosomes, their eggs, or concentrated urine from schistosome-infected patients into experimental animals has not produced lesions of importance (*58–60*).

Models of schistosomal bladder cancer in experimental animals inevitably will remain more complex than models based on the administration of chemical carcinogens. The carcinogenic stimulus presumably includes the deposition of schistosome eggs in the bladder. The sites and rate of egg laying are influenced by the varied and perhaps changing relationships between the mammalian host and the parasite. However, if a tractable model of schistosomal bladder cancer can be found, it will offer unusual opportunities to compare the human disease with a "natural" experimental counterpart.

SCHISTOSOMIASIS AND HEPATOCELLULAR CARCINOMA

S. mansoni and *S. japonicum* infections may produce hepatosplenomegaly and portal hypertension. Although clinically similar to cirrhosis in some respects, hepatocellular function is usually well preserved. The changes in the liver are characterized by pathognomonic portal fibrosis (Symmers' clay pipestem fibrosis) with maintenance of lobular architecture (*61*). Regenerative parenchymal nodules are seen in the late stages but they are generally confined to the subcapsular area (*61*). Hepatomas were equally frequent in uninfected and schistosome-infected patients in autopsy series from Brazil (*62*), Puerto Rico (*63*), Egypt (*64*), and Mozambique (*32*). In Japan, both hepatoma and circulating HbsAg were significantly more frequent in autopsied patients with *S. japonicum* than in those without, but most autopsied patients with schistosomiasis and hepatoma also had post-hepatitis cirrhosis (*65*). In Brazil, HBsAg carriers were significantly more frequent among patients with hepatosplenic schistosomiasis mansoni than among either uninfected patients or those with *S. mansoni* infection but without hepatosplenic disease (*66*). However, in this same hospital neither schistosome infection nor hepatosplenic schistosomiasis was related to cirrhosis or to hepatoma (*62*).

Equivocal increases in the frequency of hepatoma have been reported in experimental animals infected with *S. mansoni*. In mice given injections of 2-amino-5-azotoluene, hepatomas occurred earlier and much more frequently in those infected with *S. mansoni* than in those uninfected (*67*), though toxic fine structural changes in hepatocytes were less frequent and less severe than in the uninfected mice (*68*). Infected mice treated with a single dose of the antischistosomal compound hycanthone also developed hepatomas, whereas treated, uninfected mice did not (*69*). The cocarcinogenicity of *S. mansoni* infection in mice is thus a well-established fact, and under the proper circumstances a similar effect may occur in man. A marked difference exists in liver lesions in human and murine schistosomiasis. The average 2-cm² slide of liver from many, perhaps most, persons infected with *S. mansoni* contains no eggs, whereas the mouse liver is packed with them. Of approximately 200 human cases that I have examined quantitatively, only 2 had infections of intensity comparable to that in a mouse infected with a single pair of *S. mansoni* (*70*).

SCHISTOSOMIASIS AND LYMPHOMA AND LEUKEMIA

A striking association of giant follicular lymphoma of the spleen and hepatosplenic schistosomiasis was found in Brazil, 8 cases of splenic giant follicular lymphoma being found in a series of 863 surgically removed spleens (*71*). None of these patients showed peripheral lymphadenopathy, although nodes at the splenic hilum showed giant follicular lymphoma in 4 cases. One patient subsequently developed a reticulosarcoma. Another died shortly after surgery, and no residual lymphoma was found at autopsy. Several series of autopsy cases suggest an association of either lymphoma, leukemia, or both with schistosomiasis, but the nature of the association varies in the different reports (*30, 31, 72*). A suggestion of a positive association remains, and in the study from Nigeria of Edington et al. (*72*), lymphoreticular tumors were present in 16% of infected individuals and 5% of uninfected individuals.

SCHISTOSOMIASIS AND CARCINOMA OF THE COLON AND OTHER SITES

Inflammatory polyps of the colon have been frequently reported from Egypt and occasionally from elsewhere, and there is no doubt of their causal relation to schistosome infection, most frequently with *S. mansoni*. The polyps usually regress after treatment of the schistosome infection (*73*). *S. haematobium* infection frequently involves the colon and also causes inflammatory polyps (*30, 31*). The polyps do not show adenomatous changes, and cancers of the colon are not associated with schistosome infections or with schistosomal polyposis of the colon (*30, 31, 74*). Surprisingly, schistosomal polyps of the colon are rare in Brazil (*62*). Numerous case reports of coincidental schistosomiasis and colon cancer have been published, but never with evidence to indicate that cancer was more frequent in infected individuals. Extensive colon lesions occur in experimental animals infected with any of the three species of schistosome, but tumors have not been reported.

Cancers of the ovaries, uterus, and numerous other organs have also been attributed to schistosomiasis, but no evidence of more than a coincidental association has been provided.

CARCINOGENIC EFFECTS OF DRUGS USED TO TREAT SCHISTOSOMIASIS

I noted previously the cocarcinogenic effects of schistosome infection and a single dose of the drug hycanthone, a thioxanthone derivative, in the production of hepatomas in mice (*69*). In a second laboratory, no carcinogenic effect was observed (*75*). In yet a third laboratory, in which multiple drug injections were given, a carcinogenic effect was noted, but no difference was found between infected and uninfected mice (*76*). Hycanthone also has a marked mutagenic effect in the Ames test and in host-mediated mutagen assays. Using cloroindazole analogs of hycanthone, Hulbert et al. (*77*) were able to dissociate the chemotherapeutic and mutagenic activities of the drug. Several other schistosomicidal drugs show lesser mutagenic activity (*78*). Hycanthone has been used extensively in the treatment of schistosomiasis in recent years, and a related drug, lucanthone hydrochloride, was commonly used previously. Niridazole, another drug still widely used in the treatment of schistosomiasis, has been shown to produce tumors in mice on prolonged feeding. The tumors produced included transitional cell carcinomas and smooth muscle tumors of the urinary bladder (*79, 80*). Hycanthone and niridazole continue to be used in clinical practice, principally because they are much more convenient to administer than the antimonial drugs, which are also effective, and because they produce less immediate toxicity. The chemotherapy of schistosome infections has been recently reviewed by Katz (*81*).

CONTROL OF NEOPLASIA CAUSED BY SCHISTOSOME INFECTION

The control of schistosomal bladder cancer will probably be achieved through the control of schistosomiasis. Advances in the knowledge of bladder cancer are less likely to be important for the infected population than are advances in antischistosomal chemotherapy, snail control, engineering, and public health. These would have the important advantage of decreasing other morbidity and mortality caused by the infection. However, control of schistosomiasis, like the control of smoking, has proved elusive. In the meantime, better diagnosis and treatment of existing cases of schistosomal bladder cancer are important goals. Such diagnosis and treatment are not now possible for more than a small fraction of affected individuals. The annual budgets for health in developing countries are frequently 1 or 2 dollars per capita, and for the foreseeable future one cannot justify undue emphasis on treatment of the individual with bladder cancer. Elucidation of the mechanisms by which schistosomiasis causes bladder cancer will provide knowledge of great importance and general applicability, and populations exposed to *S. haematobium* infection form high-risk groups of unusual research interest.

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GUEST EDITORIAL

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The Tenth Anniversary of the Surgeon General's Report on Smoking and Health: Have we Made Any Progress?

THE SURGEON GENERAL'S FIRST REPORT on smoking, published in January 1964, linked cigarette smoking with various human diseases, particularly lung cancer, some 14 years after the first large-scale studies on the association between cigarette smoking and lung cancer had been reported. What sort of progress has been made toward the reduction of tobacco-related diseases in these 10 years? Obviously, neither the subsequent reports by the Surgeon General nor any declaration by voluntary health organizations linking smoking to health hazards would have been of practical value unless they led to a reduction of such diseases.

Preventive approaches toward the reduction of smoking-related diseases have focused on three major areas and have had varying degrees of success. Broad educational efforts have made the public increasingly aware of the relationship between smoking and the risk of disease (*1*). A considerable number of smokers have given up the habit, and this trend has been particularly evident among more highly educated men (*2*, *3*). The influence of education is less marked for women. As was expected, lung cancer death rates among exsmokers were reduced (*4–6*). The antismoking program has been less effective with young people, partially due to powerful pressure of peers who equate cigarette smoking to adulthood. Boys have maintained their rate of "taking up smoking" since 1968, whereas girls have increased their rate to equal that of the boys (*7*). Although those involved in antismoking campaigns should gain some satisfaction from the results of their work, the increasing sales of cigarettes suggest that purely informational approaches alone will not eliminate the health risks from smoking when society itself continues to condone smoking as an acceptable social habit.

The second approach to the reduction of smoking-related diseases involves smoking cessation clinics. Many cigarette smokers wish they could give up the habit but apparently are unable to do so by themselves (*1*). The latest data from the American Health Foundation's Smoking Cessation Clinic show that $\approx 25\%$ of those smoking 30 or more cigarettes per day who attended the clinic managed to stay off cigarettes for 1 year after therapy (Shewchuk L: Unpublished observations). This percentage may not be an indication of dramatic success in our efforts to find ways to help people give up smoking. If medically oriented smoking cessation clinics conducted by well-trained therapists with careful maintenance and follow-up programs throughout the country were to concentrate on those who smoke more than 30 cigarettes a day, within 10 years these efforts would surely have a significant impact on the incidence of smoking-related diseases. Additional research needs to be done on the development of drugs that will help smokers give up—and stay off—the habit. Although it is at present a relatively unexplored approach, the collaboration of the pharmaceutical industry in this regard should have a beneficial impact on the problem.

Finally, the case for the manufacture of a less harmful cigarette must be considered and work in this direction intensified. Studies of the history of tobacco usage show that, for many people, a definite "satisfaction" results from smoking. The development of tobacco products that provide satisfaction to smokers but do not damage one's health is a practical goal. Significant progress has been made during the last 20 years. Tar and nicotine levels of cigarettes produced in many countries were significantly lowered during these two decades (*8*). Studies have shown a reduction in risk of lung cancer among people who have smoked filter cigarettes for 10 years or more, compared with those who continue to smoke nonfilter cigarettes (*6*, *9*). This trend is evident even when the concomitant reduction of tar and nicotine levels in nonfilter cigarettes during this same interval is considered. These lower risks are apparent even though nearly all filter cigarette smokers in the lung cancer category started with the high tar and nicotine cigarettes of the pre-1950's.

Today's cigarettes not only yield less tar and nicotine than those of 20 years ago, but, because of changes in the manufacturing processes and the selection and blending of the tobacco used, they also yield fewer tumors in the experimental animal (*10*). At least in part as a result of this reduction in tar yield and tumorigenicity of today's cigarettes, we are beginning to see a plateau effect, if not an actual decrease, in the lung cancer incidence curves in the 35–39- and 40–44-year age groups. Similar findings should exist for other smoking-related diseases.

The "managerial" approach to the tobacco and health problem, an approach which historically has had the greatest impact on the reduction of illness, is thus starting to have an effect. At the beginning, the leveling off and subsequent decline in incidence of lung cancer and other tobacco-related diseases will be noticeable only among men. Since the percentage of women who smoke today is much higher than in 1950, a reduction in rates will not be apparent among women for some years. However, due to the modifications in the manufacture of today's cigarettes, smoking-related disease rates in women are not likely to attain the same high levels reached by men. Young people today who smoke cigarettes that yield a low smoke condensate can be expected to be at lower risk than their parents in the future.

When evaluating our progress in terms of lung cancer, we found that the U.S. death rate in 1950 was 19 per 100,000 males, and in 1971 it was 54 per 100,000 (*11;* U.S. Public Health Service, National Center for Health Statistics: Unpublished observations). It may well be said that, had no educational effort on the hazards of smoking and no work in the development of a less harmful cigarette taken place, the death rate from lung cancer might have been much higher. However, as a society, we should not rest until the rates of this and other clearly preventable diseases among smokers are reduced to the rates of those individuals who have never smoked. The recent establishment of an active, well-funded program in the Division of Cancer Cause and Prevention of the National Cancer Institute should contribute toward this end.

In conclusion, on the Tenth Anniversary of the Surgeon General's Report, it is possible to take some satisfaction from the progress made to date in the reduction of tobacco-related illnesses. Nevertheless, we need to question why society, the legislative arm of the Government, and the scientific community have not addressed this problem with the vigor, coordination, and funds necessary to combat a factor that alone contributes to more than one-third of the male cancer deaths in this country each year and also enhances the development of chronic pulmonary and cardiovascular diseases. The enactment of legislation to enable Federal regulatory agencies to set maximum tar and nicotine levels in smoking products, or, if this is not politically feasible, the voluntary setting of such levels by industry seems to be a reasonable step.

The purpose of this communication is to solicit appropriate action from all segments of society, since all of us are intensely involved with this problem and hope that on the twentieth anniversary of the Surgeon General's Report there will be better news to report about smokers, exsmokers, and nonsmokers alike. Together we must strive for the day when avoidable illnesses and disabilities will no longer be tolerated.

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GUEST EDITORIAL——————————**by Joseph F. Fraumeni, Jr., M.D.**
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Respiratory Carcinogenesis: An Epidemiologic Appraisal

Recognition of chemical carcinogenesis in man has been largely through epidemiologic studies of respiratory cancer. Although cigarette smoking is the major cause of respiratory neoplasms throughout the world, the contribution of hydrocarbons and other potential carcinogens in tobacco smoke remains to be clarified (*1*). On the other hand, industrial exposures, which account for a relatively small proportion of respiratory cancers in the general population, have been the primary means for identifying chemical carcinogens in man. It seems timely to review the epidemiologic evidence for factors other than smoking in respiratory carcinogenesis, and to note opportunities for further etiologic research.

OCCUPATIONAL AGENTS

Radioisotopes

In the past decade an increased risk of lung cancer, particularly the small-cell undifferentiated type, has been established among uranium miners in Colorado, and quantitatively related to the inhalation of radon daughter products (*2*). These agents are thought to be responsible also for the elevated risk of lung cancer among the miners of Joachimsthal and Schneeberg and the fluorspar miners of Newfoundland (*3*), and perhaps for the smaller excess risk among iron-ore miners of England (*4*) and American hard-rock miners (*5*) exposed to lower doses of radioactivity. In laboratory animals as well, respiratory tumors have been induced by inhaled radon daughters (*6*).

Mention should be made also of the upper respiratory tumors produced by the bone-seeking radioisotopes: radium and mesothorium. Ingestion of these agents by radium dial painters and radium chemists has resulted, not only in bone sarcomas but also in carcinomas of adjacent tissues, particularly the paranasal sinuses (*7*).

Mustard Gas

In 1968, Wada et al. (*8*) summarized the cancer mortality experience of Japanese workers in a factory that manufactured mustard gas in 1929–45. There were 33 deaths after 1950 from respiratory-tract cancer, compared to 0.9 expected. The tumors tended to develop centrally along the main airways (oropharynx, nasal sinuses, larynx, trachea, and major bronchus) and were either squamous or undifferentiated. Similar neoplasms have been produced by mustard gas in laboratory animals (*9*). Military exposures to mustard gas in World War I have not raised the incidence of lung cancer, probably due to the much lower dose levels than received by the factory workers (*10*).

Asbestos

Over the last 20 years, epidemiologic evidence has accumulated that the risk of asbestosis, lung cancer, and mesothelioma is substantially raised among workers in asbestos industries (miners and millers, and insulation, textile, shipyard, and cement workers) (*11*). The incidence of lung cancer varies with the extent of exposure to all types of asbestos fibers. The risk of mesothelioma is thought to be greater after exposure to crocidolite than to chrysotile or amosite, though in laboratory animals all fibers have produced mesotheliomas when injected into the pleural cavity. Also, inhalation by rats of various asbestos materials has produced lung cancer and mesothelioma. The carcinogenic activity of asbestos has been linked to the physical properties of the fibers rather than to chemical contaminants such as trace metals or hydrocarbons (*11*). Recent observations suggest that, in addition to lung cancer and mesothelioma, asbestos increases the risk of cancers arising in the larynx (*12*) and various parts of the gastrointestinal tract (*11*).

Polycyclic Aromatic Hydrocarbons

This group of compounds, highly carcinogenic to laboratory animals, is a long-recognized cause of occupational skin and scrotal cancers. Recent studies have clearly documented a lung-cancer hazard as well. In England, Doll et al. (*13*) reported excessive mortality from lung cancer among gas workers and implicated coal carbonization products. In the United States, Lloyd (*14*) reported on the mortality experience of 58,828 steelworkers, and found that men in the coke plant had a risk of lung cancer two and a half times greater than other steelworkers. The excess mortality was confined to men working at coke ovens, reaching tenfold among those employed 5 or more years at the top of the ovens. In Japan, excessive mortality from lung cancer was observed in a group of workers from a gas generator plant (*15*).

Halo Ethers

Three reports in 1973 implicated bis(chloromethyl) ether (BCME) and possibly chloromethyl methyl ether (CMME) in occupational lung cancer. In the United States, Figueroa et al. (*16*) made a 5-year survey of 111 CMME workers at a chemical manufacturing plant. Four

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Editor's note: Periodically the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

workers developed lung cancer, an incidence eight times higher than that of a comparable group of men with similar smoking histories. Subsequently, 14 other workers were identified with lung cancer, mostly oat-cell carcinomas. In Germany, Thiess et al. (*17*) observed 6 cases of lung cancer among 18 BCME workers employed in a testing laboratory. Two more cases were identified, and 5 of the 8 tumors were oat-cell carcinomas. In Japan, Sakabe (*18*) described 5 cases of lung cancer among 32 BCME workers, whereas less than 1 case (0.02) was expected.

It has not been possible to distinguish on epidemiologic grounds between the effects of these agents. CMME is usually contaminated by BCME, a highly carcinogenic substance in mice and rats after inhalation, skin application, or sc administration. The contaminated preparations of CMME are carcinogenic on sc injection in mice, but inhalation studies have been negative (*19*).

Nickel

Nasal cancer was described in 10 workers from a large nickel refining company in South Wales in the Report of the Chief Inspector of Factories and Workshops for 1932. Fifty-two cases of nasal cancer and 93 of lung cancer were reported from the refinery and classified as industrial diseases by 1950. In 1958, Morgan (*20*) and Doll (*21*) calculated that in these workers the proportionate mortality from lung and nasal cancers was significantly higher than in the general male population. In 1970, Doll et al. (*22*) reported a follow-up study of 845 men who worked at the refinery for at least 5 years. In men employed before 1925, deaths from lung cancer were about five to ten times expected. Men employed subsequently showed no excess cancer mortality, which suggested that the hazard had been removed from the refinery by 1925. A recent follow-up of men working in a Norwegian nickel refinery revealed that the risk of lung and nasal cancers was primarily in operations such as smelting, roasting, and electrolysis (*23*).

Various studies have suggested that the risk of cancer is greatest during the earliest stage of refining involving heavy exposure to dust from relatively crude ore. The view of some workers that nickel carbonyl is the culprit is inconsistent with *a*) the fading of the cancer risk, despite continued use of the carbonyl process after 1925 in South Wales (*22*), and *b*) the excess risk of respiratory cancer in refineries using the electrolytic rather than the carbonyl process in Canada and other countries (*24*). In some nickel refineries, the high levels of arsenic and other agents may influence cancer risk, but it is generally believed that nickel in some form is primarily responsible. This view is consistent with animal studies indicating that certain nickel compounds can produce local sarcomas by injection and pulmonary tumors by inhalation (*25*).

Chromium

The first demonstration of a cancer hazard among chromate workers was in 1948, when Machle and Gregorius (*26*) reported 193 deaths at six chromate-producing plants in the United States; 21.8% resulted from respiratory cancer, compared to 1.4% expected in a control group from other industries. In a survey of records from two hospitals near a chromate-producing plant, Baetjer (*27*) in 1950 found that the proportion of chromate workers among lung-cancer patients was significantly higher than that among other patients. Mortality studies on additional groups of American chromate workers confirmed an excess frequency of lung cancer. Later, in England, Bidstrup and Case (*28*) reported 12 deaths from lung cancer in a follow-up survey of chromate workers, compared with 3 expected from national death rates. The risk could not be explained by confounding variables and was ascribed to an occupational carcinogen.

An elevated risk of lung cancer has been linked also to the chromate pigment industry in Germany and, recently, Norway (*29*). The risk is not increased among chromite-ore miners but only among workers producing and processing chromium compounds. It is not clear which compounds are carcinogenic in man, although one study implicated monochromates (*26*). In laboratory animals, various chromium compounds have produced sarcomas locally by intramuscular and intrapleural injection. Epithelial lung tumors in rats have resulted from intrabronchial implantation of calcium chromate (*30*).

Inorganic Arsenic

Arsenic has not been accepted universally as a carcinogen, largely because laboratory studies have not succeeded in producing tumors in animals. However, there is little question that skin cancers in man have resulted from inorganic arsenic in drugs, drinking water, or the occupational environment (*31, 32*). Controversy has centered about lung cancer, despite evidence of elevated risk among various workers who inhale high levels of arsenic compounds. In 1948, Hill and Faning (*33*) examined the proportionate mortality of workers involved in the manufacture of sheep dip containing inorganic arsenicals and found a relative excess of deaths from cancers of the lung and skin among heavily exposed but not among unexposed workers in the factory. In 1951, Snegireff and Lombard (*34*) reported that the proportion of deaths from lung cancer among metallurgical workers exposed to large amounts of arsenic trioxide was not significantly different from that of the statewide male population. However, the mortality experience of former or retired employees from the plant was not determined, and reanalyses of the data have actually revealed a sizable excess of lung cancer in the plant workers compared with the general population (*35*). Starting in 1952, a series of autopsy studies were reported on vineyard workers from Germany and France heavily exposed to arsenical insecticides through inhalation as well as ingestion of contaminated wine (*36, 37*). Lung cancers and liver hemangioendotheliomas were found in about half the workers with cutaneous arsenicism or arsenic toxicity at death.

In 1963, Pinto and Bennett (*38*) studied 229 deaths among copper smelter workers employed from 1946–60 and distinguished arsenic-exposed and unexposed workers by the types of materials handled and by analyses of hair and urine samples. Proportionate mortality figures for the State were applied to the smelter populations; it was thought that arsenic exposure did not affect the relative frequency of deaths from respiratory cancer. The methodology of this study has been criticized, however, and a recent mortality survey by Milham and Strong (*39*) of workers from the same plant revealed 40 deaths from lung cancer, significantly higher than the 18 expected on the basis of rates in the general U.S. population.

In 1969, Lee and Fraumeni (*40*) examined the mortality experience of 8,047 smelter workers employed from 1938–63. Occupational exposures were categorized into heavy, medium, and light for both arsenic trioxide and sulfur dioxide levels. Compared with the statewide population rates, smelter workers showed a threefold excess mortality from respiratory cancer. The excess reached eightfold for employees working more than 15 years and heavily exposed to arsenic. However, the risk also increased in proportion to the degree of exposure to sulfur dioxide. Although the results suggested that inhaled arsenic is carcinogenic, an influence of sulfur dioxide or unidentified agents could not be discounted.

In 1974, a cluster of lung cancer in a Japanese town prompted a retrospective study by Kuratsune et al. (*41*). Eleven of 19 men with lung cancer (59%) had been heavily exposed to arsenic in copper smelters, compared to 15.8% in a control group. In a major development, studies of two American chemical companies producing pesticides and herbicides revealed excessive mortality rates for lung cancer and lymphoma among employees who handled arsenic[1] (*42*). In these plants, contact with arsenic trioxide and dry arsenicals was not associated with the sulfur-dioxide exposures at the metal smelters.

Elevated mortality from lung cancer has been found in additional groups exposed to inorganic arsenic, such as Rhodesian gold miners (*43*), American hard-rock miners (*5*), and workers in nickel refineries (*22, 44*), but evaluation is confounded by other suspected carcinogens in the working atmosphere. Furthermore, arsenic derived from insecticides is present in small quantities in tobacco smoke, but the suggestion (*45*) that it may contribute to the lung cancer risk in smokers is not generally held. However, the notion should not be abandoned, as the evidence accumulates that inhaled arsenic causes lung cancer in a wide variety of occupational settings.

Iron Ore (Hematite)

In 1956 Faulds and Stewart (*46*) observed that 9.4% of underground hematite miners in Cumberland, England, coming to autopsy had lung cancer, compared to 2% in comparable males from other occupations. Increased mortality from lung cancer was subsequently reported among iron-ore miners in France and Russia. In 1970, a mortality study of Cumberland miners by Boyd et al. (*4*) identified 36 deaths from lung cancer, compared to 21 expected in the local or national male populations. No excess of lung cancer was found among surface workers. The 70% excess of lung cancer in miners was attributed to iron oxide or radioactivity in the mines, or possibly to a combined effect. An influence of iron oxide, particularly in conjunction with silica exposure, would be consistent with the increased mortality from lung cancer reported among iron foundry workers, smiths, and metal grinders (*47*). However, iron oxide has not been found to be carcinogenic to laboratory animals, though hematite dust appears to enhance the carcinogenic activity of hydrocarbons on intratracheal administration (*48*). Some influence of radioactive materials is likely, in view of the excess lung cancer risk in other mining groups exposed to airborne radiation.

Wood Dust

In 1967 Acheson et al. (*49*) reported that woodworkers in the furniture industry of Buckinghamshire and Oxfordshire in England were prone to adenocarcinoma of the nasal cavity and sinuses, particularly the ethmoid. The hazard was later found in other parts of England and elsewhere in Europe and was linked also to nasal tumors other than adenocarcinoma (*50*). The risk seems to extend to various trades exposed to dust in the furniture industry and to woodworkers outside the furniture industry. The carcinogen in wood dust has not been identified.

Shoe Industry

During the English survey of nasal tumors connected with the furniture industry, Acheson et al. (*51*) detected a case cluster in Northamptonshire. Of 46 patients identified in this area, 21 were employed in boot and shoe manufacturing. The risk involved adenocarcinomas and squamous carcinomas of the nasal cavity and sinuses and was limited to workers in the dusty operations of the plant. Preliminary studies also suggest a risk among workers who repair footwear (*51*). The carcinogen remains to be isolated.

Isopropyl Oil

In the 1940's an increased risk of respiratory cancers, primarily of the ethmoid sinus, was found among workers in a U.S. plant that manufactured isopropyl alcohol (*52*). The suspected agent was isopropyl oil, the only agent in the process with tumorigenic activity in mice.

Other Agents

Certain industrial exposures have been suspected recently as causes of lung cancer, but the epidemiologic evidence is preliminary. For example, two surveys of the vinyl chloride industry have reported an excess risk of respiratory and brain tumors, in addition to the known association with liver angiosarcoma (*53, 54*). Newspaper workers heavily exposed to printing ink have also shown an elevated risk of lung cancer (*55*). Studies of textile workers have suggested an increased risk of cancers arising from the nasal cavity and sinuses (*50*) and the oral cavity and pharynx (*56*).

OTHER ENVIRONMENTAL AND HOST FACTORS

Although cigarette smokers and certain industrial workers are the major groups prone to respiratory cancer, recent developments suggest other risk factors and the importance of multifactorial causation.

Community Pollution

Industrial chemicals are not necessarily restricted to the workplace, but may cross over plant boundaries. Thus mesotheliomas have developed among family members exposed to the dusty clothing of asbestos workers and among residents in the neighborhood of asbestos industries (*57*). Low asbestos exposures may also pose a serious threat to the general public; asbestos bodies and calcified pleural plaques are present in large segments of the population, and high ferruginous body counts were found in a recent study of lung cancer patients without known occupational exposure to asbestos (*58*). Arsenic presents a similar problem; children in the neighborhood of copper smelters have urinary arsenic levels as high as the plant workers (*39*), and a recent study of lung cancer mortality revealed significantly elevated rates in men and women residing in counties with arsenic-emitting smelters (*59*). Furthermore, a survey of cancer mortality in Los Angeles County, California, revealed high rates for lung cancer in heavily industrialized areas with elevated air and soil levels of benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons (*60*). Other studies have also shown a raised incidence of lung cancer in communities with industrial pollution measured by benzo[*a*]pyrene concentrations (*61*). Further studies of the community hazard from industrial carcinogens are urgently needed.

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[1] Baetjer AM, Levin ML, Lilienfeld AM: Unpublished data.

Long suspected in the etiology of lung cancer is nonindustrial air pollution, such as produced by vehicular exhaust fumes containing hydrocarbons. Although the reported incidence of lung cancer is two to three times higher in urban than rural areas, the contribution of urban air pollution to this gradient is a matter of controversy and requires further study (*62*).

Combined Exposures

Cigarette smoking greatly enhances the risk of lung cancer after asbestos exposure. In American insulation workers, the elevated cancer risk was found to be confined to cigarette smokers, which suggested a synergistic effect (*63*). The findings were similar for men and women employed at an asbestos factory in London (*64*). There may be a synergistic interaction also between smoking and airborne radiation in the induction of lung cancer in uranium miners (*2*). The role of tobacco in other occupational cancers has not been examined satisfactorily.

Little epidemiologic attention has been given to the possible effects of combined industrial exposures. In a study of smelter workers, sulfur dioxide seemed to enhance the risk of lung cancer associated with arsenic exposure (*40*). Similarly, in rats, inhalation of the carcinogen, benzo[*a*]pyrene, combined with the irritant sulfur dioxide, produced squamous-cell carcinomas of the lung; inhalation of the carcinogen alone did not produce tumors (*30*).

Alcohol and Nutrition

Alcohol consumption predisposes to cancers of the larynx, as well as oropharynx and esophagus, and seems to act synergistically with cigarette smoking (*65*). Experimental studies suggest that alcohol is not carcinogenic by itself, but in some manner enhances the effect of tobacco and perhaps other agents.

A recent dietary survey of men with lung cancer revealed a negative association with an index of vitamin A intake (*66*). This preliminary finding is consistent with the results of animal experimentation (*67*). Further study of nutritional factors may help to explain the elevated risk of lung cancer in the lower socioeconomic class and in people who migrate from rural to urban areas (*68*) and from the South to the North (*69*).

Drugs

Although respiratory tumors result primarily from the direct action of inhaled carcinogens, some agents may localize secondarily in the respiratory tract. Radiochemicals have produced nasal–sinus carcinomas, not only by *a*) direct instillation of Thorotrast as a radiopaque medium (*70*) but by *b*) diffusion from bone into sinus cavities of radium and mesothorium after medicinal or occupational exposures (*7*). Patients receiving intravenous Thorotrast are at increased risk of liver hemangioendothelioma and leukemia, but the early suggestion of a raised incidence of lung cancer could not be confirmed (*71*). Potassium arsenite, once used orally for psoriasis and other diseases, caused chronic skin changes, with pigmentation, hyperkeratoses, and cancer (*31*). Clinical surveys of treated patients with arsenical dermatoses have suggested an excess risk of lung cancer (*72*), but epidemiologic confirmation is lacking.

Certain alkylating agents are carcinogens in man. Chlornaphazine produced bladder tumors, and melphalan and cytoxan seem to be leukemogenic (*73*). At autopsy, several patients given certain agents such as Myleran (busulfan) have shown cytologic abnormalities of a precancerous nature in the lung and other sites. In one patient the lung changes seemed to progress to bronchiolar cell carcinoma (*74*). Immunosuppressive agents may predispose to lung cancer, according to a recent follow-up survey of recipients of kidney transplants. In addition to a substantial excess risk of histiocytic lymphoma (*75*), we found 5 new patients with lung cancer, mainly adenocarcinoma, compared to 1.8 expected from the experience of the general population.[2]

Genetic and Immunologic Susceptibility

Tokuhata and Lilienfeld (*76*) reported a significant tendency for lung cancer to aggregate in families and a synergistic interaction between familial predisposition and smoking. Recently, Kellermann et al. (*77*) found that lung-cancer patients have increased levels of inducible aryl hydrocarbon hydroxylase activity in cultured lymphocytes. The findings suggest a genetically regulated enzyme defect that affects the metabolism of hydrocarbons, so in time it may be possible to identify individuals especially prone to respiratory carcinogens in cigarette smoke or polluted air. In addition, studies of nasopharyngeal carcinoma suggest that the predisposition of Chinese to this tumor is linked to certain antigens of the HL-A system (*78*).

Clinical studies indicate that occupational lung diseases, particularly from coal dust, are severe in patients with rheumatoid arthritis (rheumatoid pneumoconiosis or Caplan's syndrome). Also, persons with various pneumoconioses, including asbestosis, have an increased frequency of rheumatoid factor and other autoantibodies in their sera (*79*). These findings suggest that autoimmune states accelerate the development and progression of chemically induced lung diseases, some of which predispose to cancer. However, among the connective-tissue diseases, only scleroderma (progressive systemic sclerosis) seems prone to lung cancer (adenocarcinoma and alveolar-cell types) (*80*).

The relation of immunodepressive states to lung cancer is suggested by some recent observations: *a*) Kidney transplant recipients receiving immunosuppressive drugs are prone to lymphoma and probably to lung adenocarcinoma[2]; *b*) in immunodepressed mice, the incidence of spontaneous lung adenomas is raised (*81*); *c*) subclinical immune defects have been found in a family with multiple cases of lymphoproliferative neoplasms and lung adenocarcinomas (*82*); *d*) sarcoidosis patients, who have impaired cellular immunity, are reportedly prone to lymphomas and lung cancer (*83*); *e*) patients with asbestosis tend to have impaired cellular immunity (*84*) and histocompatibility antigens (*85*) that might affect the subsequent risk of neoplasia; and *f*) patients with early lung cancers have defective cellular immunity (*86*), which may have a function in carcinogenesis. On the other hand, lung cancers have not developed excessively in the primary immunodeficiency syndromes or in disorders other than transplantation treated with immunosuppressive drugs.

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[2] Hoover R, Fraumeni JF Jr: Unpublished data.

FUTURE STUDIES

Newer understanding of respiratory carcinogenesis in man will come not only from increased epidemiologic efforts in testing existing hypotheses but also from developments in various fields generating fresh etiologic hypotheses that can be pursued by epidemiologic study.

Experimental Studies

Animal and other laboratory studies may predict human responses and provide leads to carcinogenic hazards that should be pursued promptly by epidemiologic studies. Although nearly all human carcinogens produce tumors in laboratory animals, only a limited number of carcinogens in animals have been shown to cause neoplasms in man, perhaps because the doses used in experimental studies are so much higher. Thus knowledge that beryllium produces lung and bone tumors in experimental animals prompted surveys of beryllium workers, but as yet no excess risk of cancer has been established (*87*). Isoniazid, used extensively for tuberculosis, produces lung adenomas in laboratory animals, but surveys in man have shown no cancer hazard (*88*).

On the other side of the coin, laboratory studies should be conducted promptly whenever clinical or epidemiologic observations suggest new carcinogenic exposures. The mounting evidence implicating inorganic arsenic as a human carcinogen, for example, should stimulate experimentalists to apply new techniques to evaluate carcinogenicity in laboratory animals.

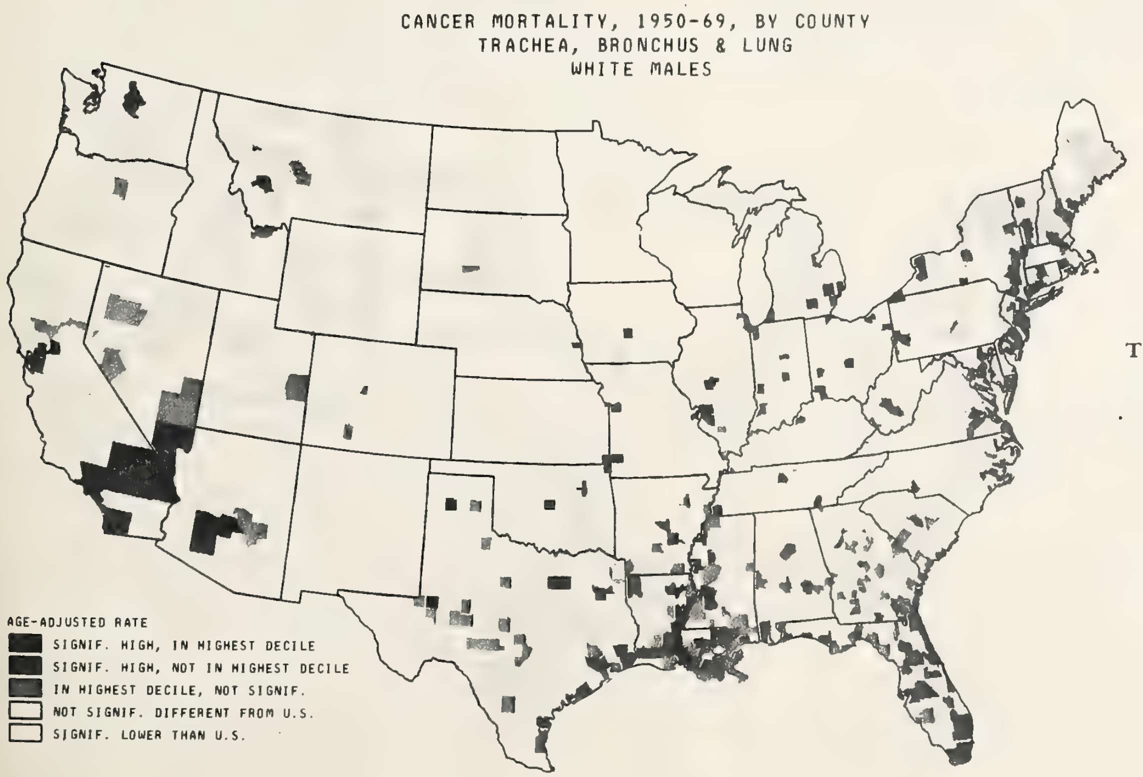
Whenever possible, the interdisciplinary approach is desirable in studies of human cancer and high-risk groups, with experimental methods used that measure environmental exposures, genetic susceptibility, or other risk factors identifiable on a subclinical level.

Clinical Observations

Over the years the initial leads to environmental carcinogens have been supplied mainly by alert clinicians, especially by reporting clusters of cancers ordinarily rare in the population at large. In respiratory cancer, a relatively common disease, suspicions may be heightened if the patients in the series have common environmental exposures, are unusually young or light smokers, have tumors with a peculiar site or cell-type distribution (e.g., ethmoid sinus cancers, mesotheliomas, oat-cell neoplasms), or show associated toxicity (e.g., dermatoses and nasal perforation from arsenic, and pneumoconioses from asbestos, chromium, or nickel). To encourage clinical leads and ensure epidemiologic pursuit, consultation should be available to practitioners by national and international agencies concerned with cancer etiology.

Demographic Leads

Clues to environmental factors may come from the monitoring of cancer incidence and mortality statistics. In the United States, ethnic differences are provocative, such as the elevated risk of lung cancer in black men (*89*) and in Mexican-American (*90*) and Chinese women (*91*). Cancer registries are striving toward prompt recognition of upturning rates that might signal the introduction of new environmental hazards (*92*). In a new development, U.S. cancer mortality statistics have been systematically analyzed and mapped on a county level at the National Cancer Institute (*93, 94*). The lung-cancer pattern for white males has revealed clustering in the Northeast and in a series of counties along the southeast Atlantic and Gulf coasts (text-fig. 1). In these southern rural areas, the contribution of environmental factors other than smoking is probably much greater than in the rest of the country, so that field studies in high-risk communities are more likely to uncover carcinogenic factors. To help focus the search, the cancer patterns are being correlated


CANCER MORTALITY, 1950-69, BY COUNTY
TRACHEA, BRONCHUS & LUNG
WHITE MALES
AGE-ADJUSTED RATE
■ SIGNIF. HIGH, IN HIGHEST DECILE
■ SIGNIF. HIGH, NOT IN HIGHEST DECILE
▨ IN HIGHEST DECILE, NOT SIGNIF.
☐ NOT SIGNIF. DIFFERENT FROM U.S.
☐ SIGNIF. LOWER THAN U.S.

TEXT-FIGURE 1.—Mortality from lung cancer by U.S. county among white males, 1950–69.

with industrial and demographic data available from the Census Bureau and other agencies. For example, in counties where chemical industries are highly concentrated, men were found to have excessive mortality from certain cancers, particularly the lung, bladder, liver, nasopharynx, and nasal sinuses (*95*). This resource is being used also to test hypotheses regarding community hazards. For example, the cancer mortality patterns were not unusual in Colorado counties where residents were exposed to uranium mill tailings used as construction fill (*96*), or in a Minnesota county where the drinking water was contaminated with asbestos (*97*).

Registries

When experimental, clinical, or demographic observations point to a possible cancer hazard, steps should be taken rapidly to assemble a case series for retrospective evaluation of occupational and other risk factors. This approach would be greatly enhanced by the systematic acquisition of occupational and other epidemiologic data by cancer centers and by population-based cancer registries in industrialized parts of the country. In addition, the screening of death certificates for occupational data is a simple and valuable method of formulating hypotheses to risk factors. Critical to the detection of cancer risk and elucidation of carcinogenic exposures are occupational cohort studies; a continuing assessment of at-risk groups should be made to determine the best bets for epidemiologic investigation.

Environmental hazards may be detected also as a by-product of surveys of diseases suspected to be at a high risk of cancer. For example, a study of multiple primary tumors in Birmingham, England, revealed that men with scrotal cancer were prone to cancers of the lung and upper digestive tract (*98*). Since the scrotal tumors were primarily in mineral oil workers, the new tumors might have been caused by oil mist in the atmosphere. Similarly, the carcinogenic potential of immunosuppressive and cytotoxic agents may be clarified by surveys of patients with organ transplants (*75*), connective tissue disorders, adverse drug reactions, and various neoplasms included in treatment-evaluation programs.

Record Linkage

Since the respiratory tract of workers in many industries is heavily exposed to specific airborne pollutants, there is every indication to believe that well-designed occupational studies will continue to be the most rewarding means of identifying risk factors in respiratory carcinogenesis. Newer methodologies are needed, however, to reduce the complexity, expense, and length of time involved in these studies. In various countries, on a regional or national basis, special attention should be given to the possibility of record-linkage systems for matching individual records of employment with records of cancer registration and death certificates. The collaboration of industry, unions, government officials, and medical organizations should be sought in development of the data resources and techniques that will permit a systematic monitoring of occupationally related cancer.

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ADDENDUM

Although cigarette smoking is the major cause of lung cancer in the United States, the influence of other environmental determinants was suggested by a county-by-county survey that showed elevated mortality rates among white men in certain parts of the country, especially along the Gulf of Mexico and along the southeast Atlantic coast (*1*).

Occupational factors have been suspected, since correlation analyses indicate elevated mortality in counties with certain manufacturing industries, e.g., chemical and petroleum manufacturing (*1*, *2*) and shipbuilding (*3*). These findings have helped determine the location and questions to be asked for a series of interview studies of patients with lung cancer and controls in an effort to identify reasons for the elevated rates. In a recent study in coastal Georgia, Blot et al. (*4*) reported a significantly increased risk associated with employment in the area shipyards that operated during World War II and closed soon after the War's end. The summary relative-risk estimate was 1.6 after adjusting for smoking, other occupations, age, race, and county of residence. The increased risk was not limited to any single trade within the industry, such as insulators or pipe coverers who have heavier exposures to asbestos. However, a synergistic interaction was noted between shipyard employment and tobacco smoking, consistent with the multiplicative effects previously described in asbestos workers (*5*, *6*). These findings suggest that asbestos and possibly other shipyard exposures during wartime employment account for part of the excess mortality from lung cancer in certain coastal areas of the country.

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EDITORIAL

by John J. Mulvihill, *M.D.*[1]

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Host Factors in Human Lung Tumors: An Example of Ecogenetics in Oncology

Conventional approaches to understanding the etiology of lung cancer in man have focused profitably on environmental agents *(1)*. Efforts to identify and control pulmonary carcinogens have been most successful with hazards of occupational inhalants such as radioactive ores, mustard gas, asbestos, nickel, chromates, arsenic, and certain petrochemicals *(2)*. In contrast, the carcinogenic mechanisms of the commonest human carcinogen in the general population, tobacco, are poorly characterized and poorly controlled through public education *(3)*. Pending development of less hazardous cigarettes and advances in motivational research, identification of host factors that modify susceptibility to known respiratory carcinogens is timely.

The term "ecogenetics" may be applied to heritable variations in susceptibility to environmental agents. An appreciation of human ecogenetics avoids the deceptive dichotomy of considering environmental agents to the exclusion of genetic determinants in cancer etiology. Exploration of ecogenetics may lead occupational health workers to identify susceptible individuals and may contribute to further knowledge of carcinogenesis.

DEMOGRAPHIC VARIABLES

Standard epidemiologic data give few clues to host-related factors *(4)*. Rapid changes in the *incidence* of lung cancer and its *sex* ratio are well explained by trends in tobacco use and not by large changes in mutation rates or gene pools *(5)*. The *geographic distribution* of lung cancer in the United States suggests variations from occupational exposures, except for a concentration in rural southern Louisiana, where inbreeding is conspicuous *(6)*. Compared to whites, the incidence of lung cancer in the U.S. is accelerating more rapidly in blacks *(7)*, is higher in Americans of Mexican and Chinese extraction *(8, 9)*, and is lower in American Indians *(10)*. Worldwide *ethnic* and geographic variations might arise from genetic and environmental factors, which can sometimes be distinguished by studying the cancer experience of migrants. For lung cancer, the observations are inconclusive: The *migrant* rates seem intermediate between the rates in their former and new countries *(11)*. Chinese in Hong Kong have a peculiar predominance of adenocarcinoma of the lung, a relatively infrequent cell type elsewhere *(12)*.

Age relates strongly to certain cell types. Bronchogenic carcinoma is seldom seen in children and young adults *(13, 14)*. In 9 years, the U.S. Childhood Cancer Mortality Registry ascertained only 60 primary lung tumors in persons under 20 years of age (Miller RW: Unpublished data). No carcinomas were reported in children under age 5 years (when prenatal origins may be suspected); all were sarcomas, teratomas, and hemangiomas.

Studies of *twins* have not shown a genetic factor because of inadequate numbers of cases, though one study of 10,945 twin pairs was large enough to demonstrate the influence of smoking on lung cancer *(15)*.

EMPIRIC FAMILIAL RISK

Where demography falls short, a more powerful tool, the case–control study, has shown significant familial influences that could be environmental, genetic, or both. In two separate studies, Tokuhata and Lilienfeld *(16, 17)* found 2.4 and 2.7 times more lung cancer among relatives of patients with lung cancer than among relatives of controls. The familial factors were more evident among nonsmokers than among smokers. Conversely, the effect of smoking was less evident among case relatives (who had the familial tendency already) than among control relatives. The familial and smoking effects were synergistic. These data *(16, 17)* were not analyzed by histologic pattern, but lung cancer is a heterogeneous disease with a variety of cell types and suspected etiologies. As Anderson has done for breast cancer *(18)*, studies of specific pathologic types of lung cancer might reveal strong genetic factors that are undetectable when all cell types are combined.

PEDIGREE ANALYSIS

Two reported sibships with squamous cell carcinoma of the lung suggest an interaction between external agents and genetic susceptibility *(19, 20)*. In both families, the patients with lung cancer had been heavy smokers, a trait well demonstrated to have strong familial tendency in both twin and case–control studies *(15, 16)*. In one family (text-fig. 1A), the eldest brother worked in a copper smelter; the youngest affected brother had

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ABBREVIATIONS USED: IPF=interstitial pulmonary fibrosis; AHH=aryl hydrocarbon hydroxylase; MEA=multiple endocrine adenomatosis.

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Editor's note: Periodically, the Journal publishes editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

exposure to chromium fumes *(20)*.

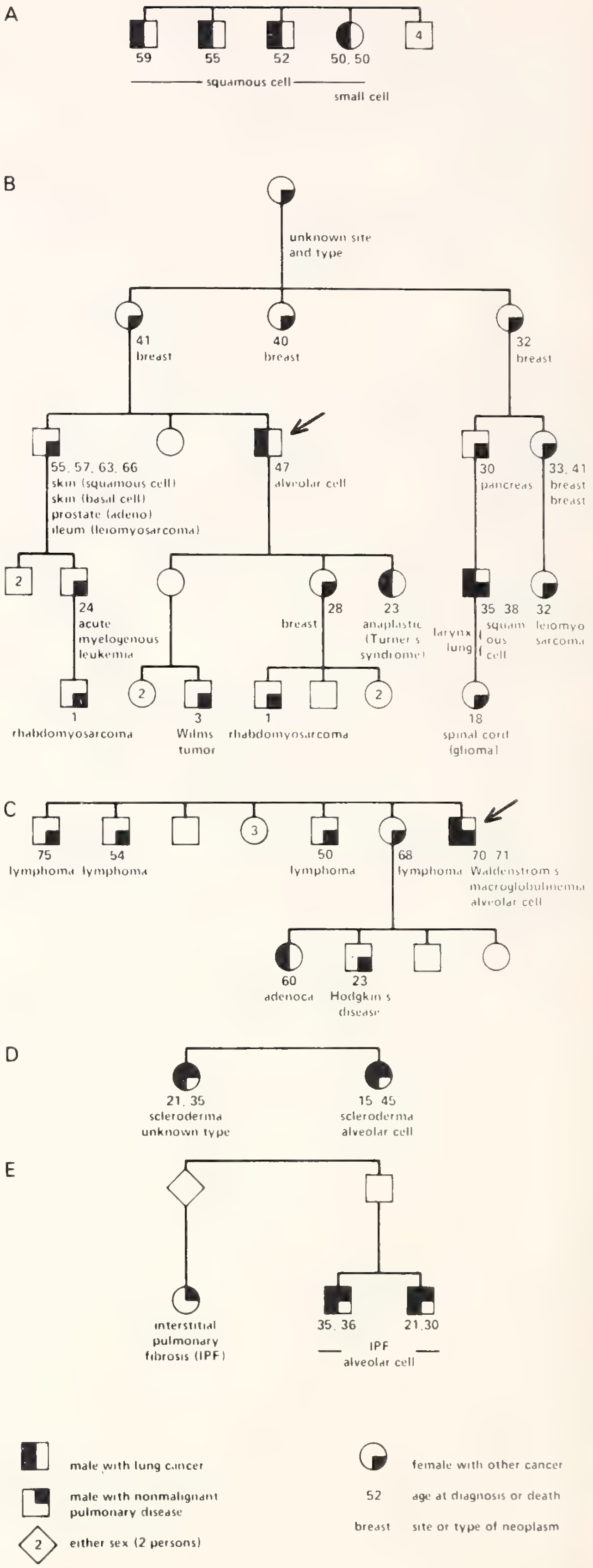
Lung cancer is occasionally seen in families that seem to have an excess of tumors of diverse cell types. It appears in pedigrees with excessive ovarian and breast cancers *(25)*. In one family [A in original report *(21)*] with the familial soft-tissue sarcoma–breast cancer syndrome of Li and Fraumeni, a man (text-fig. 1B, *arrow*) developed alveolar cell carcinoma of the lung, his daughter with Turner's syndrome had anaplastic lung cancer, and a distant cousin had metachronous squamous cell carcinomas of the larynx and lung (Dettmer CM: Personal communication). In another family studied at NIH, alveolar cell carcinoma and pulmonary adenocarcinoma developed in a man (text-fig. 1C, *arrow*) and his niece; in addition, they and many first-degree relatives had lymphoproliferative disorders, seen clinically or only as subclinical B- and T-cell dysfunction *(22)*.

MULTIPLE PRIMARY NEOPLASMS

Families B and C in text-figure 1 and others *(25)* suggest that lung cancer may occur as a second primary neoplasm more often than expected by chance alone, though the frequency of pulmonary metastases makes proof of an independent primary tumor in the lung especially difficult. Among all patients with multiple primary neoplasms in the Connecticut Tumor Registry *(26)*, the risk of developing lung cancer was significantly elevated in men with cancer of the lip, mouth, pharynx, and larynx, and in women with cervical cancer. For men, the risk may be attributed to continued use of tobacco, which may also contribute to the risk in both sexes after bladder cancer. [The converse strengthens this possibility: Lung cancer patients have a significant risk for subsequent cancer of the mouth and upper respiratory tract *(27)*]. Finally, lung cancer was reported in association with antimetabolite therapy for cancer *(28)* as well as for psoriasis *(29)*.

IMMUNE FACTORS

In addition to these case reports *(28, 29)*, scattered evidence is converging that relates immune defects to the genesis of some lung tumors: *a)* In two series of lung cancer patients, B- *(30)* and T-cell *(30, 31)* functions were depressed at diagnosis. However, in 42 patients with multiple primary neoplasms (including 21 with lung cancer), the numbers of T-lymphocytes and distribution of HL-A types were normal *(32)*. *b)* Uranium miners, known to be at high risk for radiogenic lung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15 45
scleroderma
alveolar cell
E
interstitial
pulmonary
fibrosis (IPF)
35, 36
21, 30
IPF
alveolar cell
male with lung cancer
male with nonmalignant pulmonary disease
2
either sex (2 persons)
female with other cancer
52
age at diagnosis or death
breast
site or type of neoplasm

TEXT-FIGURE 1.—Examples of familial aggregations of lung cancer. A) Squamous cell carcinoma of the lung in a sibship of smokers; the oldest and youngest affected brothers also had occupational exposure to respiratory carcinogens *(20)*. B) Diverse neoplasms including lung cancer with the familial soft-tissue sarcoma–breast cancer syndrome of Li and Fraumeni *(21)*. C) Pulmonary adenocarcinoma with clinical and subclinical lymphoproliferative disease *(22)*. D, E) Alveolar cell carcinoma with familial scleroderma *(23)* and IPF *(24)*.

tumors, had normal immunoglobulin levels at the time of employment; these levels fell after 1 year and remained low even 10 years later *(33)*. *c)* Lung cancer has occurred in 8 renal transplant recipients on immunosuppression, compared to 1.8 expected (Hoover R, Fraumeni JF Jr: Unpublished data). *d)* Intensive drug and radiation therapy for extrapulmonary cancer regularly produces atypia of alveolar linings *(34)*. *e)* Certain disorders with immunologic abnormalities may be associated with lung cancer, such as dermatomyositis, sarcoidosis, and scleroderma. A strong heritable tendency has been observed in each condition except dermatomyositis, which seems to be a paraneoplastic manifestation of lung cancer and not a disease predisposing to it *(35)*. In 2,544 patients with sarcoidosis seen in 10 years, 9 lung tumors were observed compared to 2.8 expected *(36)*.

By 1975, over 25 patients with scleroderma (progressive systemic sclerosis) had alveolar cell carcinoma of the lung *(37)*. Striking features of the association are the 7:1 female:male ratio (compared to 2–3:1 for scleroderma in general) and positive correlation with the intensity of IPF. The observed progression of fibrosis to lung cancer in scleroderma is likely to be a common route by which various disorders predispose to cancer, especially alveolar cell carcinoma *(38)*. This concept of pathogenesis, similar to that suggested for hepatocellular carcinoma after cirrhosis, unites under the rubric of "scar carcinoma" such diverse pneumonic processes as pulmonary infarction, rheumatic mitral valve heart disease, and desquamative interstitial pneumonia. In these conditions, intermediary alveolar metaplasia and benign adenomatosis are frequent autopsy findings, and the tumors are thought to be multifocal in origin. In addition to the immunologic defects, the finding of excessive chromosome breaks in the peripheral blood and bone marrow of patients with scleroderma supports its malignant potential *(39)*. Breaks were seen also when cells from normal controls were preincubated in sera from affected patients. Although another study related the breaks to cyclophosphamide therapy *(40)*, scleroderma may join the group of chromosome fragility syndromes that have a high risk of cancer *(41)*.

ANTECEDENT HERITABLE LUNG DISEASE

Scleroderma may explain certain familial aggregations of lung tumors such as the sisters reported by Petitjean et al. *(23)*; one of these had alveolar cell carcinoma (text-fig. 1D). Another sibship of 2 brothers with alveolar cell carcinoma (text-fig. 1E) was probably explained by the concomitant familial diffuse IPF (present also in a cousin without tumor) *(24)*. An autosomal dominant form of congenital cystic disease of the lung (familial fibrocystic pulmonary dysplasia), indistinguishable from Hamman-Rich syndrome except by its chronicity and inheritance, was associated with lung cancer in two brothers *(42)* and with sporadic lung tumors in members of other affected families *(43, 44)*.

Other autosomal dominant traits have IPF as an occasional feature and could be associated with lung cancer, though I know of no reports. These syndromes include certain phakomatoses (neurofibromatosis, tuberous sclerosis, and von Hippel-Lindau disease) *(45)*, Marfan's syndrome *(46)*, and familial pulmonary alveolar microlithiasis *(47)*.

As for autosomal recessive traits, the occurrence of pulmonary liposarcoma in 1 of 4 sisters with congenital adrenal hyperplasia is probably a coincidence *(48)* but may be iatrogenic, since hyperadrenocorticism can cause large mediastinal lipomas *(49)*. Multiple leiomyoma, sometimes a recessive trait when it affects the skin *(50)*, can also involve the lungs *(51)*. A 52-year-old man was reported with Kartagener's syndrome and bronchogenic carcinoma *(52)*, again probably fortuitous despite the presence of immunodeficiency and pulmonary malformations that may predispose to lung cancer in other disorders.

In one Swedish family, autosomal dominant inheritance of IPF and an abnormal hemoglobin (Malmö) was associated with anaplastic small cell carcinoma *(53)*. Although a subsequent American family with abnormal hemoglobin (Malmö) had no pulmonary disorder *(54)*, in the Swedish family this hemoglobin can be considered a genetic marker for lung cancer.

GENETIC MARKERS, INCLUDING AHH

A more controversial marker is the inducibility of AHH. AHH represents a multiple component enzyme system that catalyzes endogenous and exogenous lipid-like molecules to more polar ones for better elimination. In experimental systems it also converts polycyclic hydrocarbons, as found in cigarette smoke, to intermediate metabolites that are potent mutagens and carcinogens. AHH is found in human lungs (specifically in alveolar macrophages), in peripheral monocytes *(55)*, and in most organs. In man, cigarette smoking can induce sustained elevation of AHH *(56)*. In mice, inducibility varies in many inbred strains—in some as an autosomal dominant, in others as a codominant trait *(57)*.

Relating AHH to human lung cancer, Kellermann et al. *(58, 59)* of the M.D. Anderson Hospital and Tumor Institute, Houston, Texas, reported two simple but stunning observations in 1973. First, AHH inducibility in man seemed to be controlled by a single locus with two alleles *(58)*. Second, in a series of patients with lung cancer, 96% (compared to 55% in controls) had high or intermediate levels of inducibility; low levels were found in 4% of lung cancer patients but in 45% of controls *(59)*.

For several reasons, the early promise of these findings has faded after 3 years of relative silence. First, simple Mendelian inheritance is unlikely in man because AHH is not one but several enzymes and because codominance occurs in inbred mice. Second, evidence for one locus with two alleles comes from a frequency distribution of inducibility that was said to have three modes *(58)*, but the small number of cases makes the interpretation tenuous. Third, the Houston assay depended on lymphocytes stimulated by mitogens and might not reflect the metabolic activity of bronchial mucosa. Furthermore, T-cell function is depressed in many lung cancer patients *(30, 31)* who would thus be unavailable for the Houston assay *(58)* because their lymphocytes do not transform. In mice, lymphocytic transformability appears to be a heritable trait *(60)*. Finally, one-fourth of the 50 Houston patients had adenocarcinoma, a cell type not clearly related to smoking.

Certainly, a biochemical or genetic marker of cancer susceptibility would be a welcome advance. Although the relation of AHH inducibility to human lung cancer may be debatable, there is no better candidate trait. Genetic deficiency of α_1-antitrypsin predisposes to emphysema of the *panlobular* type *(61)*, and lung cancer occurs to excess in patients with emphysema but of the opposite variety, namely *centrilobular (62)*. In any case, α_1-antitrypsin levels are increased in patients with lung cancer *(63)*. As for other genetic markers, Terasaki and Mickey *(64)* reported a significant excess of HL-A haplotypes A1 and A7; blood group B has been associated with adenocarcinoma, and group O with undifferentiated cancer of the lung in males *(65)*.

CONCLUSIONS

In lung cancer as in most human disease, pathologic heterogeneity signals important etiologic differences. In bronchogenic carcinoma, for example, cigarette smoking is the most important etiologic factor, and little evidence exists for inherited influences. On the other hand, numerous peculiarities of adenocarcinoma and alveolar cell carcinoma suggest familial factors: They are poorly related to smoking, are found in families with other tumors, acquired immune diseases, or heritable disorders of the lung (especially those associated with IPF and scleroderma), and they occur as an autosomal dominant trait in mice *(66)*. Alveolar cell carcinoma in sheep is suspected to be a viral disease called jaagsiekte *(67)*.

For even rarer cell types, genetic factors may emerge when sufficient cases are assembled. The syndromes of MEA include pulmonary neoplasms such as bronchial mucosal neuroma in MEA III and bronchial carcinoid in MEA I (Wermer's syndrome) *(68)*. Carcinoid alone can be an autosomal dominant trait and shares many histologic, histochemical, and endocrinologic features with oat-cell carcinoma *(69)*.

In the search for additional genetic factors, the area to examine may be the interaction of known environmental carcinogens with susceptible genotypes *(70)*. This field might be termed ecogenetics, a word already used to apply to the long-term effects of the environment on the genome, but it may tolerate a special use in medical genetics. By analogy to pharmacogenetics, the study of variations in the response to drugs, clinical ecogenetics could be the study of variations in the response to environmental agents in general. One challenge in ecogenetics in oncology might be the development of means for screening potential employees for abnormal genotypes that predispose them to neoplasia after occupational exposures that are harmless to normal genotypes.

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ADDENDUM

A later version of this summary appeared in (*1*). Ecogenetics continued to emerge slowly as a comprehensive perspective for considering the origins of human cancer (*2, 3*).

Demography

Black Ohio residents with lung cancer had a twofold risk of being born in the South rather than in Ohio; this intracountry migrant effect suggested lung cancer determinants in early life, perhaps genetic or dietary (*4*). Ethnic comparisons in Texas showed that, compared with whites, rates were higher in Mexican Americans over age 74 but much lower in those under 70 (*5*). The peculiar excess of adenocarcinoma of the lung in Cantonese females of Singapore was not associated with smoking, but with low consumption of green vegetables, and implicated susceptibility due to relative deficiency of vitamin A (*6*).

Pedigrees

In a sibship of 12 adults, 2 sisters developed adenocarcinoma and a brother had squamous cell carcinoma; all were smokers and 39–43 years of age (*7*). Reports of lung cancer and pleural mesothelioma in spouses and children of asbestos workers emphasized the familial hazards of chemicals brought home on work clothes (*8, 9*). Peculiarities of alveolar cell carcinoma continued to appear, e.g., a report of the tumor in identical twin brothers with similar ages of onset, histochemistry, and metastatic behavior (*10*). An extensive pedigree of the familial soft-tissue sarcoma–breast cancer syndrome of Li and Fraumeni (*see* text-fig. 1B of the Editorial) confirmed the suspicion that respiratory cancer is a feature of the syndrome (*11*). Finally, lung cancer has been reported in families with retinoblastoma (*12*).

Antecedent Lung Disease

Case reports appeared of peculiar lung tumors associated with congenital malformation of the lungs: undifferentiated carcinoma with ipsilateral hypoplasia of the pulmonary artery (*13*); rhabdomyosarcoma in congenital cystic adenomatoid malformation of the lung (*14*); and anaplastic carcinoma in a fibromyomatous hamartoma of the lung (*15*).

Genetic Markers

Aryl hydrocarbon hydroxylase remained a prime but controversial candidate for a genetic marker of lung cancer. Twin studies suggested significant genetic determinants (*16*). Each study with findings of higher inducibility in lymphocytes of lung cancer patients, compared with controls (*17–19*), seemed balanced by negative reports (*20–23*). While difficulties with the lymphocyte assay were debated, others turned to assays of pulmonary alveolar macrophages (*20*) or explants of bronchial mucosa (*24*).

No unusual α_1-antitrypsin phenotypes were detected in 72 patients with lung cancer compared with 196 controls (*25*). Serum activity was doubled, consistent with the previous interpretation [*see* (*62*) in Editorial] that genetic α_1-antitrypsin deficiency does not predispose to lung cancer as it does to emphysema. Lung cancer and chronic obstructive pulmonary disease may still have a common familial factor. First-degree relatives of patients with each disease have similarly impaired forced expiration, a pulmonary function test, compared with controls with nonpulmonary disease (*26*). In 12 "lung cancer families," a history of chronic obstructive pulmonary disease was found in more first- than second-degree relatives (*27*).

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Information on (*26*) of the Editorial can now be completed.

(*26*) SCHOENBERG BS: Multiple primary malignancies: The Connecticut experience. Recent Results Cancer Res 58:1–173, 1977

by Arnold E. Reif[1]

JNCI 57:1207–1210, 1976

Public Information on Smoking: An Urgent Responsibility for Cancer Research Workers

A MAJOR "BREAKTHROUGH" IN CANCER RESEARCH?

The newspapers are always hunting for a new breakthrough that will drastically reduce cancer in our population. It seem ironic that the most meaningful breakthrough in cancer research likely to occur during our lifetime has already been made. Its very simplicity may prevent us from recognizing it for what it is.

This paper outlines the evidence that one quarter of all cancer deaths in the United States are caused by smoking and suggests ways in which any cancer research worker who so desires can devote spare time to community service toward prevention of these deaths.

EXCESS CANCER DEATHS FROM CIGARETTE SMOKING

Leaders in medical research in Great Britain, the United States, and many other countries have identified tobacco smoking as by far the most effective carcinogenic stimulus in our environment of which we are aware, in terms of the number of citizens adversely affected. According to Lord Zuckerman (*1*), "Lung cancer, which now accounts for about 25% of the total cancer mortality in the United Kingdom, is largely a preventable disease, and that action which reduced cigarette smoking would be more immediately effective in reducing mortality from lung cancer than that aimed at finding a cure for the established condition."

A recent U.S. Public Health Service report (*2*) states that "Cigarette smoking has been clearly identified as the major cause of lung cancer in the United States." This conclusion is based on detailed epidemiologic, clinical, autopsy, and experimental data accumulated over more than 20 years. For both men and women, the risk of developing lung cancer is directly related to the total exposure to cigarettes, as measured by statistics such as age at initiation of smoking, duration of smoking, lifetime total number of cigarettes smoked, depth of inhalation of the smoke, and tar levels of cigarettes smoked. According to the editor of *Science* (*3*), 30% of all cancer in males in America is due to cigarette smoking. Just how true are these statements?

For those who appreciate being able to examine for themselves how such figures are derived, a statistical appendix is included. The data indicate that 23.5% of *all* cancer deaths in the United States during 1974 were caused by smoking. The data available at this time suggest that this figure rose above 25% for 1975.

LOGISTICS OF PREVENTION VERSUS CURE OF LUNG CANCER

Because lung cancer often metastasizes early and has a relatively short course, present statistics on the cure of lung cancer are dismal. Would the introduction of a reliable test for early detection of lung cancer vastly improve these statistics?

The answer to this question is given by two studies on the effect of frequent (semiannual) use of a battery of clinical tests for early detection of lung cancer. In a British study (*4*), the 5-year survival rate for those who developed lung cancer was 15% for 101 new cases in the screened group of 30,000 men. In an American study (*5*), the 5-year survival rate was 12% for 67 men whose lung cancers were detected within 6 months of a negative X-ray examination and 4% for 54 men whose lung cancers were detected beyond this time. Thus even when a high-risk group (elderly male smokers) received semiannual screening, the 5-year survival rate was only about 8.5% better than without screening. In studies without screening, survival runs between 4 and 8% (*6*) and depends on the particular histologic type and site of origin of the tumor (*7*).

Suppose a reliable test for early diagnosis of lung cancer were developed that was sufficiently superior to the above methods (*4, 5*) to double the increase in survival obtained by semiannual screening. How many lives would it save? The limiting factor would be the motivation of the right segment of our population to submit to such a test often enough to realize the potential saving in life. A realistic estimate of the people at high risk who could be motivated to take such a test might be 10% of their number. Under these circumstances, the actual savings in life would be only 10% of 17%, or less than 2% of current lung cancer deaths. Therefore, discovery of a new test for early diagnosis of lung cancer would not appreciably affect lung cancer death rates.

In contrast, cessation of cigarette smoking can eliminate all but a small percentage (<10%) of the excess risk of lung cancer (*7, 8*). If ceasing to smoke can prevent lung cancer, then never smoking is obviously more effective. The scientific validity of the causation of lung cancer from smoking is now as firmly established as any link between cause and effect involving human beings can ever be (*1–3, 6–9*).[2] The logistics of prevention are simply that 81% of lung cancer deaths could be avoided if our population did not smoke (*see* "Appendix", table 1). Because advances in diagnosis of lung cancer could improve its cure rate only by perhaps 2% (*see above*), the odds are approximately 40 times higher in favor of work on the prevention of this unnecessary disease.

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[2] The author played a small part in this story by being able to obtain for the first time unanimous agreement between research workers at the Seventh International Cancer Congress in 1958 that cigarette smoking was the main cause for the then current increase in lung cancer, which was a controversial statement at that time (*9*).

Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

EXCESS DEATHS FROM SMOKING OTHER THAN THOSE DUE TO CANCER

Cigarette smoking is presently the most important cause of chronic bronchitis and emphysema, diseases that spell long years of incurable ill health. Further, "In the United States, coronary heart disease (CHD) is the leading cause of death and the largest contributor to excess deaths among cigarette smokers. Cigarette smoking acts independently of and synergistically with the other CHD risk factors to greatly increase the risk of developing CHD. The risk of developing CHD for pipe and cigar smokers is much less than it is for cigarette smokers, but more than it is for nonsmokers" (*2*). When the mortalities from all types of death were determined, those for cigarette smokers were 64% higher than for nonsmokers; in contrast, the overall age-related mortalities for cigar and pipe smokers were only 16 and 11% higher, respectively (*2*). The distribution of excess deaths for cigarette smokers (compared with nonsmokers) between the three main categories of smoking-caused diseases, namely cancer, chronic bronchitis plus emphysema, and cardiovascular disease, is approximately 1:1:5. Finally, data both from prospective and from retrospective epidemiologic studies have documented a relationship between maternal cigarette smoking and spontaneous abortion (*2*).

WHY PARTICIPATE IN EDUCATION ON SMOKING?

Research intended to alleviate human ills remains unfulfilled unless research findings are transferred to, and applied in, community practice. If not carried over to everyday life, research can be accused of being little more than a titillating exercise for the scientific intelligentsia. In this sense, every research worker, whether or not he or she will acknowledge it, is involved in the social responsibility to ensure that research findings are taught in his or her local community. If the responsibilities of the physicists who discovered atomic fission carried over into the public sphere, as they did for Albert Einstein and many others, then our responsibilities as cancer research workers also do not end in the laboratory. If public information agencies have trouble in persuading society to act on and adopt our research findings, who is better able to help than we? Who is better informed about the meaning of our findings? We who make our livelihood from cancer research, should we be too busy to serve our own community with our specialized knowledge?

For many cancer research workers, such as myself, laboratory work with experimental animals gives one little evidence of direct (rather than potential, long-term) contribution toward prevention, detection, or cure of cancer in man. Participation in public education within one's community gives a welcome indication that cancer research has made vital contributions to human well-being, and permits one to become involved with people rather than with experimental animals.

HOW TO ENTER PUBLIC EDUCATION ON SMOKING

For years, I would have been happy to do something useful for my community in the area of cancer education, if someone had asked me; no one did. Perhaps readers can identify. Finally, I realized that if I were really serious about becoming involved, I would have to take the initiative.

In my case, the trigger was a film, available free of charge from the National Institutes of Health, for high school audiences. Entitled "Getting Through," it starred Burt Lancaster and could be obtained for two weeks at a time. To see what would happen if I offered my services to show the film and discuss it, I telephoned the principal of my sons' high school in the suburban Boston community of Wellesley and asked him if he would like me to come. His response was enthusiastic, and within 5 minutes the time for an assembly of the 10th grade class, over 500 strong, had been arranged. Encouraged, I called the principals of eight other Boston and suburban high schools and experienced a similarly positive response. I scheduled assemblies during the first period of the day, so that I would be only slightly late to work on days on which I gave assemblies.

Because such assemblies seemed to fill a real need, I contacted other cancer research workers, thoracic surgeons, and physicians whose previous involvement in assembly work had come to my attention. At a meeting the next spring (May 1969), we formed a loose society, which, a year later, became *Volunteers for Health Awareness*. Our intent was to give every youngster in the Greater Boston area the opportunity to hear the full story of the effects of smoking at least once. To arrange assemblies, we divided the Greater Boston area into five districts, with a coordinator for each district. For new speakers, I produced a canned lecture with 25 slides; this remains freely available to anyone who would like to use it. In time, most speakers modified their presentations.

Seven years later, Volunteers for Health Awareness has over 20 active members. Its purpose is "to disseminate health information, especially to the young, with emphasis on self-injurious practices." Anyone who shares the group's views on such practices may join; no dues are required. Members give over 50 assemblies each year mostly to seventh and eighth grade students, covering nearly half of all junior high schools in the Greater Boston area. Members have been active in local radio and television talk shows, especially during the annual National Education Week on Smoking and Health, which is arranged by the Interagency Council on Smoking and Health for the second week in January of each year. Our members have also been active in local city and State legislation in the area of smoking in public areas, and have coordinated publicity in the local newspapers.

OTHER ORGANIZATIONS ENGAGED IN PUBLIC EDUCATION ON SMOKING

My suggestion to cancer research workers or physicians who would like to become active in public education on smoking is to get together with friends of like mind and discuss plans for involvement. This allows considerably more freedom of action than if one were to put one's services at the disposal of local societies that are run by paid nonmedical staffs. However, collaboration with such societies can be rewarding, and several recently formed volunteer organizations are well worth joining.

The *American Cancer Society* has for many years led the battle to give the public the true facts concerning smoking, even faced with an expertly managed public relations campaign by the tobacco industry, which has long sought to discredit the link between smoking and disease. However, the American Cancer Society has many other worthwhile goals (such as the early diagnosis of different types of cancers), which prevent it from a single-minded campaign against smoking. More recently, the *Heart Association* has been a staunch ally in public informaton on smoking. Now that tuberculosis has succumbed to improved nutrition, public health, and chemotherapy, the *Lung Association* has begun to place major emphasis on air pollution, whether of the outdoors, or of the indoor environment where the chief source is smoking. The Lung Association has recently sponsored the formation of citizens' chapters of *Fresh Air for Nonsmokers*, with the goal of introducing local and State legislation on nonsmokers' rights, and public information on the effect of tobacco smoke on nonsmokers.

The government office concerned with public information on smoking is the *National Clearinghouse on Smoking and Health*[3], which has performed invaluable service by its yearly updating of the "1964 Surgeon General's Report on Smoking and Health." More than any other factor, the publication of the original report can be credited with reversing the increasing per capita cigarette consumption in this country. The Clearinghouse operates a Technical Information Center on smoking and its effects on health that has become an international resource for researchers and others interested in this area. It has produced scores of attractively designed brochures and films for public distribution (as have the American Cancer Society and the Heart and Lung Associations). It is a backhanded tribute to the effectiveness of the Clearinghouse, that its Congressional appropriation for the fiscal year 1974 was somehow "forgotten"; undoubtedly, the Clearinghouse is a thorn in the sides of legislators from tobacco-growing states. It recently suffered a severe dislocation by a move from Bethesda, Maryland, to Atlanta, Georgia. This was the fourth move forced on it in 7 years.

The Interagency Council on Smoking and Health was formed to coordinate the antismoking activities of the many (33) voluntary and government agencies that have similar interests. From the cancer researcher's or physician's viewpoint, collaboration with the local, State or citywide Interagency Council (if one exists) is fine. However, one's activities should not be restricted to a few brief "campaigns" that the local staffs of the major voluntary agencies (such as the cancer, heart and lung associations) may agree to undertake in concert.

Action on Smoking and Health was founded in 1968 by John F. Banzhaff III, a Washington, D. C., attorney who is Executive Director. Banzhaff is responsible for a petition filed with the Federal Communications Commission that resulted in the ruling that the "Fairness Doctrine" must be applied to cigarette advertising, i.e., that radio and television stations must devote substantial air time to antismoking messages. Later, Action on Smoking and Health filed a complaint with the Federal Trade Commission that charged the tobacco industry with unfair and deceptive trade practices. This eventually triggered Congressional action resulting in the law that ended cigarette advertising on the electronic media as of January 1, 1971. The organization has also been instrumental in limiting smoking on airlines and supporting strengthened health warnings on cigarette packages. New chapters have recently been formed in a number of States, and from my own experience with the Massachusetts chapter (in which I am research secretary) I can thoroughly recommend this society.

Another valuable local voluntary organization is *Smokers Anonymous*,[4] which was started by a thoracic surgeon, Richard Overholt, with Alcoholics Anonymous as a pattern. Run by ex-smokers and a few physicians or medical scientists in Boston, the group conducts withdrawal clinics that meet once weekly for 10 weeks. It would be a challenge to start similar volunteer groups in other locations.

In contrast, *The 5-Day Plan to Stop Smoking* is available in many cities of the United States, as a nonprofit service of the staffs of Seventh Day Adventist hospitals. The clinics consist of sessions on 5 successive days, and claims are (in common with Smokers Anonymous) that half the attendees quit smoking for at least several months. A few commercial firms such as *smokEnders* are active in several States, as are some hypnotists, in trying to help those who so desire to stop smoking.

HOW EFFECTIVE IS PUBLIC EDUCATION ON SMOKING?

Since the 1964 Surgeon General's report, some 30 million Americans have stopped smoking, according to the American Cancer Society. When a local radio station (WBZ) put on a massive antismoking campaign with the help of members of Volunteers for Health Awareness in 1970, they received over 6,000 "quit smoking" pledges by mail, probably only a fraction of those who quit as a result of this campaign. Anonymous questionnaires filled out by over 3,400 seventh and eighth graders 4–12 weeks after one of my assemblies showed that over 29%

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[3] *Address*: Center for Disease Control, Building 14, Atlanta, Ga. 30333.

[4] *Address*: 135 Francis Street, Boston, Mass. 02215.

Table 1.—*Excess cancer deaths caused by cigarette smoking in the United States in 1974*

Type of cancer	Deaths in 1974 D	Fraction of smokers f	Mortality ratio m of smokers to nonsmokers (reference)	Excess deaths due to smoking	
				E	%
Bronchogenic[a]	78,873	0.43[b]	11 (*11*)	63,966	81.1
Laryngeal	3,262		5.5 (*11*)	2,150	65.9
Oral[c]	7,968		4 (*11*)	4,494	56.4
Esophageal	6,652		4.5 (*11*)	3,998	60.1
Bladder	9,397		2 (*12*)	2,819	30.0
Pancreatic	17,376		2 (*13*)	5,213	30.0
Renal	7,073		2 (*12–14*)	2,122	30.0
Total	130,601			84,762	64.9
All types of neoplasms[d]	360,472			84,762	23.5

[a] Includes carcinoma of trachea, bronchus, and lung.

[b] Smoking in U.S. adults in 1965: Past history influences cancer incidence (*10*).

[c] Includes malignant neoplasms of buccal cavity and pharynx.

[d] Includes benign neoplasms and lymphatic and hematopoietic malignant neoplasms.

of current smokers had quit smoking after the assembly and had not started again at that time. This indicates a potent initial impact, at least.

While it is easy to question the long-term results of any public information attempt, there is one positive aspect of the involvement of scientists in public affairs which seems beyond debate: Research scientists cannot help doing research in any area in which they become interested. Hopefully, this will lead to new information and new methods of attack on old problems.

Finally, I should add that involvement in this area of public education has been an enjoyable and rewarding experience for me. For instance, at perhaps every second assembly I give to very young people (seventh graders), one or two of them will approach me afterwards and shyly thank me for coming. That's reward enough for me. In your community, it's up to you.

APPENDIX: Estimation of Excess Deaths due to Cigarette Smoking

The following data illustrate the means by which estimates of excess deaths due to smoking are made. The mortality figures are the latest available from the National Center for Health Statistics.[5] To estimate the number of excess deaths due to cigarette smoking, I have derived the following equation:

$$E=\frac{D\cdot f(m-1)}{1+f(m-1)}$$

where E=excess deaths due to cigarette smoking, f=fraction of smokers in the population, m=mortality ratio of smokers to nonsmokers for a specified type of cancer, and D=number of deaths from that type of cancer.

This equation considers the number of smokers who would have developed cancer of the respective site in the absence of smoking. The value of f is an average value over the years relevant for the final deaths, typified by the figures for 1965 when 52% of males and 34% of females smoked (*10*). Some degree of uncertainty with regard to the exact value of the mortality ratio m must remain unresolved. When this ratio is high, as in the case of bronchogenic cancer, the difference in number of deaths attributed to smoking varies surprisingly little with variation in m. Incidences for bronchogenic carcinoma are still rising: The American Cancer Society has estimated that there will be 93,000 new cases in 1976 (*15*). Table 1 indicates that 23.5% of all cancer deaths in 1974 were excess deaths due to cigarette smoking. A similar computation for 1974 restricted to males (not tabulated here) indicates that 32.7% of all cancer deaths represent excess deaths due to cigarette smoking. Whereas the data on lung cancer for 1975 are not yet complete, there was a 6% increase over 1974 in deaths from malignant neoplasms of the respiratory system.[5] This large increase strongly suggests that by 1975 at least one quarter of all deaths from cancer in our population were unnecessary deaths caused by cigarette smoking.

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[5] *Address*: 5600 Fishers Lane, Rockville, Md. 20852.

ADDENDUM

During the 2 years following publication of the above Editorial, the incidence of cancers caused by cigarette smoking has continued to rise, especially in women. When cancers caused by cigar and pipe smoking are included, the percentage of human tumors caused by smoking is even higher than reported above. This Addendum includes the effects of cigars and pipes and updates the previous data.

A few months after publication of the Editorial, I received a letter from Dr. Morton L. Levin, who told me that he had published in a specialty journal long ago (*1*) a slightly different form of the same equation that I had included. He called this equation a formula for calculating attributable risk, which he defined as the proportion of a disease in the general population attributable to a given etiologic or associated factor (*1*). Since then, at least two other scientists published unintentional rediscoveries of his equation. Recently, Levin simplified his equation (*2*). Also, a formula for calculating the variance of attributable risk was developed (*3*). I am delighted to avail myself of this opportunity to set the record straight.

To calculate excess deaths caused by smoking, I used the same method as in table 1 above. This corrects for cancer deaths of any given site that would have occurred in the absence of smoking. The values obtained (table 1 below) do not include excess cancer deaths in exsmokers and therefore represent underestimates of the true situation.

For men, excess deaths due to cigar and pipe smoking add significantly to those caused by cigarettes. The total excess deaths from smoking represent, respectively, 97, 99, 78, and 83% for cancers of the lung, larynx, oral cavity, and esophagus (table 1 below). For tumors of the

TABLE 1.—*Excess deaths caused by smoking in the United States during 1978*

Type of cancer	Deaths estimated for 1978[a]	Cigarette smokers			Cigar smokers			Pipe smokers			All smokers	
		Mortality ratio[b]	Excess deaths *E*	Excess deaths %	Mortality ratio	Excess deaths *E*	Excess deaths %	Mortality ratio[c]	Excess deaths *E*	Excess deaths %	Excess deaths *E*	Excess deaths %
Males only[d, e]												
Bronchogenic	70,500	11	56,112	79.6	2.4	5,125	7.3	4.2	7,283	10.3	68,520	97.2
Laryngeal	2,900	5.5	1,849	63.8	7.7	791	27.3	3.5	239	8.2	2,879	99.3
Oral	5,950	4	3,208	53.9	4.6	998	16.8	3.3	455	7.6	4,661	78.3
Esophageal	5,200	4.5	3,006	57.8	5.2	990	19.0	2.7	300	5.8	4,296	82.6
Bladder	6,900	2	1,936	28.1								
Pancreatic	10,900	2	3,058	28.1								
Renal	4,600	2	1,291	28.1								
Total	106,950		70,460	65.9		7,904	7.4		8,277	7.7	86,641	81.0
All tumors	213,500		70,460	33.0		7,904	3.7		8,277	3.9	86,641	40.6
Females only[d]												
Bronchogenic	21,900	11	16,285	74.4								
Laryngeal	450	5.5	255	56.6								
Oral	2,450	4	1,140	46.5								
Esophageal	1,900	4.5	957	50.4								
Bladder	3,000	2	674	22.5								
Pancreatic	9,100	2	2,046	22.5								
Renal	2,800	2	629	22.5								
Total	41,600		21,986	52.8								
All tumors	176,500		21,986	12.5								
Males plus females												
Bronchogenic	92,400		72,397	78.4							84,805	91.8
Laryngeal	3,350		2,104	62.8							3,134	93.6
Oral	8,400		4,348	51.8							5,801	69.1
Esophageal	7,100		3,963	55.8							5,253	74.0
Bladder	9,900		2,610	26.4							2,610	26.4
Pancreatic	20,000		5,104	25.5							5,104	25.5
Renal	7,400		1,920	26.0							1,920	26.0
Total	148,550		92,446	62.3							108,627	73.1
All tumors	390,000		92,446	23.7							108,627	27.9

[a] Values represent American Cancer Society estimates of deaths in 1978 (*4*).

[b] Ratio = deaths in smokers to deaths in nonsmokers. Sources are the same as in table 1 of Editorial.

[c] Mortality ratios are the mean values taken from summaries of prospective etiologic studies [chapt 6, tables 20, 22, 24, and 26, for oral, laryngeal, esophageal, and bronchogenic cancer, respectively) of cancer in nonsmokers and in smokers (*5*)].

[d] Values are fractions of smokers of age 20 and older in the United States population in 1975 (National Clearing House on Smoking and Health, DHEW: Personal Communication). Fraction *f* for male and female cigarette smokers = 0.39 and 0.29, respectively.

[e] Values are fractions of smokers of age 21 and older in the United States population in 1970 [chapt 6, table 1 (*5*)]. Fraction *f* for male cigar and pipe smokers = 0.056 and 0.036, respectively.

seven sites listed, smoking is responsible for over 80% of cancer deaths. Over 40% of all tumors in men are caused by smoking.

For women, deaths from lung cancer are rising at a faster rate than for any other type of cancer: three-quarters of these deaths are due to smoking. When both men and women are considered, nearly three-quarters of the cancers of the seven affected sites are caused by smoking. Altogether, 28% of all cancer deaths in the United States could be avoided by not smoking (table 1).

The present data (table 1) indict smoking even more seriously than those in the original Editorial. Smoking of all types inflicts so much suffering as its end result that to make it socially unacceptable seems appropriate. This would happen *rapidly* if every nonsmoker would make it a habit to ask (*gently*) each of his smoking acquaintances whether they are aware of their insensitivity to the well-being of their body and to the pollution of the environment.

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GUEST EDITORIAL——by Ernest L. Wynder and Bandaru Reddy

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Studies of Large-Bowel Cancer: Human Leads to Experimental Application [1]

LARGE-BOWEL CANCER constitutes a major cause of cancer deaths in the Western countries; however, it has been given little attention by the experimental oncologist. Perhaps the reason is that no carcinogens which relate to human large-bowel cancer have been identified. The idea of working with feces may have discouraged potential investigators; also, histologic and biochemical leads are not as easily derived from bowel mucosa as they are from sections of other organs. Whatever the reason, the incidence of large-bowel cancer continues unabated and largely unresearched.

Epidemiologic and laboratory investigations must be pursued to find the cause(s) of large-bowel cancer in man, so that the disease may be prevented. Such efforts are important, since we have not been able to significantly reduce the death rate from colon cancer, though the reported rate of deaths from rectal cancer has declined.

Since the most important leads to the etiology of any disease come from epidemiologic studies, we should review the available information in this field.

Epidemiologic Leads

Many researchers have recently investigated large-bowel cancer (*1–6*). The mortality ratios of cancer of the colon and cancer of the rectum differ so widely around the world that possible differences in etiology are suggested. Data from these epidemiologic studies are summarized.

Demography data.—1) The mortality rate from cancer of the colon is higher in the Western world than in Japan and other developing countries (*7*). 2) The mortality rate from cancer of the rectum is as high in Japan as in the United States (*7*). 3) The mortality rate from cancer of the colon increases rapidly among Japanese immigrants to the United States (*8*). 4) In the United States, colon cancer has a male:female ratio of about 0.8:1 before menopause, after which the ratio approaches 1:1. By contrast, rectal cancer has a male:female ratio of about 1.6:1 before menopause, after which it declines to 1.2:1 (*6*, *7*). 5) There has recently been a slight increase in mortality from colon cancer and a slight decrease in that from rectal cancer in the United States (*9*). These changes in incidence rates may be real or may result from recent improvements in diagnostic skills (*10*, *11*).

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Identified etiologic factors.—Retrospective studies with interview techniques have primarily succeeded in screening differences between patients with large-bowel cancer and control patients; they have not isolated or identified factors relating solely to colon cancer (*6*). In an epidemiologic survey, there are at least 3 possible interpretations: 1) No risk factors exist; 2) the population has an even distribution of the risk factors; 3) the interview technique does not identify the risk factors; and/or 4) the relevant risk factor is not asked.

Diet is a good example of categories 2 and 3, since it may be similar among individuals of a given population group, particularly in the United States, and may be difficult or impossible to quantify accurately by retrospective interview techniques. Furthermore, a particular food item cannot be implicated, since the intake of one food may be related to the consumption of another. Excessive intake of carbohydrates, for instance, is likely to be associated with a lowered intake of fats; a high intake of meat may be related to a high intake of other foods, a diet indicative of affluent living except in those societies in which meat is readily and cheaply available and other foods are not.

Ulcerative colitis (*12*, *13*) and familial polyposis (*14*, *15*) have been consistently associated with cancer. Among the negative or inconsistent variables are bowel habits (*6*) (particularly constipation and use of purgatives), obesity, smoking, use of alcohol, and previous medical history. We found that the reported relationship of appendectomy to large-bowel cancer must be artifactual (*16*). We are currently trying to substantiate a report linking cigarette smoking with polyps of the colon (*17*).

Although present studies of diet in the United States probably will show no positive results, a study of Japanese patients suggested that increase in the rate of colon cancer is associated with the recent Westernization of the diet and with a rising standard of living (*18*). Also, colon cancer was more common in higher socioeconomic groups which probably ate more Western foods. This would agree with the known increase in colon cancer among Japanese in the United States who shifted to American eating habits (*8*).

Studies of the diet of population groups, rather than of individuals, yield more reliable results. People in areas with a high incidence of colon cancer also consume large amounts of fat and animal protein. This suggests that the risk of colon cancer may be affected by the composition of the diet. The major differences between American and Japanese diets are total calories and intake of fats, proteins, and simple carbohydrates (*19*, *20*). Although a particular food may harbor a carcinogen or procarcinogen, the total diet probably carries a carcinogenic or procarcinogenic potential.

The high-fat diet common in northern Europe and North America affects the production and composition of neutral sterols and bile acids. It may also influence the intestinal bacterial flora that degrade or modify the fecal bile acids and neutral sterols. These factors might have an etiologic effect on cancer of the colon (*6*, *18*, *21–23*). Burkitt incriminated simple sugars and low-bulk, refined diets as the central causes of colon cancer because of their influence on bacterial flora (*3*, *4*). The worldwide distribution of colon cancer correlates, however, far better with fat and type of protein intake than with consumption of sugar and other highly digestible, easily dissolved carbohydrates.

Further studies of intestinal transit time are important. Although Burkitt's data indicate a shorter transit time for Africans than for European people, probably the contents of the lower gut per se are of main etiologic significance in large-bowel carcinogenesis. Transit time primarily reflects dietary intake. Furthermore, the length of transit time reported for Africans seems sufficient for bacterial and chemical conversion of intestinal contents to result in a carcinogenic environment.

We also stress that the studies on Western population groups have not related the frequency of bowel movements to large-bowel cancer (*6*). Since constipation in Western populations is far more frequent in women, one would expect a higher frequency of this disease in women than in men, if transit time were important in large-bowel carcinogenesis.

Generally, researchers agree that diet relates to colon cancer, either because of its influence on bacterial flora or its effect on the constituents of the intestinal content that become fecal matter. Hill et al. have reported some intriguing leads (*21–23*). Comparing fecal constituents from countries with various rates of colon cancer, they showed a strong correlation between high concentrations of neutral sterols and bile acid derivatives in the feces and the incidence of colon cancer. Lacassagne et al. found that some bile acids were sarcomagenic (*24*, *25*). The theoretical basis for the carcinogenic activity of bile acids and neutral sterols is based on their steric similarity to such carcinogenic hydrocarbons as 3-methylcholanthrene (MCA) and many naturally occurring sterols, the chemical conversion of deoxycholic acid to MCA, and the possibility that some microflora could perform this conversion.

These findings form the basis of our research hypothesis.

Experimental Leads

Manipulation of diet can change the composition of bile. Variations in the intake of fats, proteins, and carbohydrates have produced a marked change in pool size and production of bile acids secreted in bile as well as changes in the excretion of fecal bile acids and neutral sterols in man and animals (*26*). As animals become domesticated (e.g., the wild versus the domestic rabbit or the wild versus the domestic pig), the composition of their bile becomes more complex. It would be pertinent to compare the bile makeup and metabolites, bile acids, and neutral sterols in feces of Japanese and of Americans.

Our preliminary data show lower values for bile acids and their metabolites and for cholesterol and its metabolites in the feces of Japanese and American vegetarians. These data agree with those of Hill and his co-workers (*21–23*). Also cholesterol gallstones, particularly those consisting of bile acids, are less common in Japan than in the United States, but they seem to be becoming more frequent in Japan (*27*).

We also emphasize that biologically active compounds other than bile acids may be involved in the etiology of colon cancer. These compounds include ammonia and select amines (major products of urea and protein degradation), and indole and tryptamine formed by microbial action on tryptophan (*28*). Little is known about the quantitative importance of microbial reactions that produce ammonia and tryptophan metabolites from nitrogenous substrates, but the quantity of these compounds depends on the qualitative and quantitative aspects of intestinal microflora and also on the nature and quantity of substrate available to the flora. The incidence of colon cancer and the fecal concentration of these compounds may be correlated. Several components and substances produce large-bowel cancer in various animal species (table 1).

The key questions are whether any of the compounds in table 1 are carcinogenic in humans, and whether any of the results in animals approximate the human response. Although there is no current evidence that man is exposed to any of the carcinogens studied, carcinogenic polycyclic aromatic hydrocarbons (PAH) could theoretically be formed in the gut from precursors in the feces. The large bowel of laboratory animals is, furthermore, susceptible to this type of carcinogen (*38*, *39*). In this way *N*-nitrosopiperidine could be formed in vivo from nitrite and piperidine in the small intestines of rats (*40*), and methylnitrosourea could be produced in their stomachs (*39*).

In our studies of experimental PAH-induced tumors, we attained a yield of approximately 1 tumor in 30 rats given topical intrarectal application of BP, DMBA, and MCA. Although, in an experimental setting, these results are low, a 1 of 30 tumor yield in man would be extremely high. In our evaluation of experimental results we must constantly be aware of the health problem that comparable results in man would represent.

Cycasin, another experimental carcinogen, demonstrates the involvement of lower gut bacterial flora in its carcinogenic activity. Neoplastic changes in various organs and the intestinal tract of laboratory animals have been reported after the animals were fed crude cycad materials containing this glycoside (*41*, *42*). While cycasin is not carcinogenic when fed to germfree rats (*43*), methylazoxymethanol, the aglycone of cycasin, is toxic and carcinogenic to conventional and germfree rats (*44*, *45*). Thus for cycasin to be toxic and carcinogenic, it must be hydrolyzed to its aglycone

TABLE 1.—*Induction with chemical carcinogens of many large-bowel tumors in animals*

Author	Year	Species* [strain]	Compound†	Route‡	Induction time (average days)	Dosage	Results
Walpole et al.(*29*)	1952	R [Albino]	4-Aminobiphenyl.	sc	570	2 mg/100 g body wt/day.	Colorectal tumors in 7 of 23 rats.
Walpole et al.(*29*)	1952	R [Albino]	3,2′-Dimethyl-4-aminobiphenyl.	sc	220	2 mg/100 g body wt/day.	Intestinal tumors in 22 of 24 rats.
Della Porta(*30*)	1961	H [Syrian golden].	MCA	ig	310	5 mg/animal/week	Cecum-colon tumors in 24 of 50 hamsters.
Laqueur(*31*)	1965	R [Sprague-Dawley; Osborne-Mendel].	Cycasin	Feeding	294	2%, 1.5%, 200 ppm, 400 ppm of diet.	53 colorectal tumors in 37 of 144 rats.
Spjut and Spratt(*32*).	1965	R [Wistar]	3,2′-Dimethyl-4-aminobiphenyl.	sc	220	2 mg/100 g body wt/day.	Colon tumors in 26 of 78 rats.
Druckrey et al.(*33*)	1967	R [BD]	1,2-dimethylhydrazine.	sc	333	7 mg/kg body wt/week.	Colorectal tumors in 22 of 26 rats.
Druckrey(*34*)	1970	R [BD]	Azoxymethane	sc	500	2 mg/kg body wt/week.	Colorectal tumors in 24 of 32 rats.
"	1970	R [BD]	Azoxymethane	sc	500	12 mg/kg body wt/week.	Colorectal tumors in 3 of 6 rats.
"	1970	R [BD]	Methylazoxybutane.	sc	500	30 mg/kg body wt/week.	Colorectal tumors in 10 of 28 rats.
"	1970	R [BD]	1,2-Dimethylhydrazine.	po	206	21 mg/kg body wt/week.	Colorectal tumors in 17 of 27 rats.
Spatz(*35*)	1970	H [Syrian golden].	Methylazoxymethanol.	iv	—	Single dose 0.9–1.2 mg.	Colon tumors in 27 of 35 hamsters.
Narisawa et al.(*36*)	1971	R [Donryu]	MNNG	ir	±350	1.2 mg in 0.5 ml/day for 32 days.	Colorectal tumors in 7 of 9 rats.
Homburger et al.(*37*).	1972	H [Inbred]	MCA	po	±350	5 mg in 0.5 ml oil, 3× wk for 17 wk.	50% colon cancer in strain 15.16 males; 65% in males and 33% in females of strain 87.20.
Weisburger (*5*)	1972	R [Fischer F/344].	1,2-dimethylhydrazine or azoxymethane.	sc	±200	0.1–0.2 mmole 1× wk for 10, 16, or 20 wk.	Various diets, high or low in protein; high yield of colon cancers.

Reddy (unpublished).	1972	R [Fischer CD/germ-free].	1,2-Dimethylhydrazine.	sc		10 mg/kg body wt/wk.	In progress; injections will continue for 20 weeks. Animals will then be held for observation.
Narisawa (unpublished).	1973	R [Donryu]---	Methylazoxymethanol.	ir	-------------------------------		
So et al. (unpublished).	1972	R [Sprague-Dawley].	3,2′-Aminobiphenyl.	sc	±160–190	100 mg/kg body wt in 0.5 ml peanut oil/wk.	Tumors: mammary, 29; small intestine, 13; ear, 16; colon, 2; heart, 2; ovary, 2; cecum, 1; kidney, 1; sarcomas (including 1 chlorosarcoma), 7.
"	1972	R [Sprague-Dawley; M [Ha/ICR].	3,2′-Aminobiphenyl.	sc	±160–190	50 mg/kg body wt	Tumors: lung adenoma, 6; fibrosarcoma at injection site, 4; malignant lymphoma of lung, 5; mammary, 1; amyloidosis of spleen, 2; pyelonephritis, 4; liver sarcoma, 3; amyloidosis of liver, 4; malignant lymphoma of liver and pancreas, 1; thymus enlarged, 1; malignant lymphoma of liver, 2; granuloma of skin, 1; carcinoma of lung, 1; malignant lymphoma of spleen, 2.
"	1972	R [Sprague-Dawley].	MNNG--------	ir	±240	2 mg/kg body wt in 1.0% CMC 2× weekly for 35 weeks.	106 adenomas in 30 animals.
Wynder et al. (unpublished).	1972	R [Sprague-Dawley].	BP------------ DMBA. MCA (in 1.0% CMC).	ir	±435	Various doses between 50 and 500 mg 1 and 2× weekly.	1 colon tumor in 30 animals. 1 sarcoma, 1 colon tumor in 30 animals. 1 colon tumor in 30 animals.
"	1972	H [Syrian golden].	BP------------ DMBA. MCA (in 1.0% CMC).	ir	±435	Various doses between 50 and 500 mg, 1 and 2× weekly.	1 mammary tumor in 60 animals. No tumors. 3 sarcomas, 3 liver tumors, and 1 mammary tumor in 60 animals.
"	1972	H [Syrian golden].	MCA------------	ig	±510	Various doses between 7.5 and 15 mg in 15 ml peanut oil 1 and 2× weekly.	1 skin cancer, 4 small-intestine tumors, 19 mammary tumors, 1 papilloma on forestomach, 2 sarcomas, 1 colon tumor in 90 animals.

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*R=rat; H=hamster; M=mouse.

†MNNG=*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; BP=benzo[*a*]pyrene; DMBA=dimethylbenz[*a*]anthracene; CMC=sodium carboxymethyl cellulose.

‡sc=subcutaneous; ig=intragastric; po=oral; iv=intravenous; ir=intrarectal.

through the activity of β-glycosidase of bacterial origin (*44*).

Here, more than in experiments with simple squamous epithelia or simple connective tissues, we should ask: Is there any form of lower animal that can be used meaningfully in large-bowel cancer studies?

To determine whether any of the animal systems can be related to man, we must consider the composition of the bile acids, the overall bile flow, the bacterial flora, the constitutents of the feces, and the ability of the animals to adjust to different types of diets. Studies should evaluate whether lower animal species can mimic the human system as it relates to the etiology of colon cancer.

Proposed Studies

We are exploring the carcinogenicity of the expected or established large-bowel carcinogens (DMBA, BP, MNNG, and dimethylhydrazine dichloride) for rats, mice, and hamsters given high-fat or low-fat diets of animal or vegetable origin. 1,2-Dimethylhydrazine is also being tested on germfree animals. These studies include biochemical examination of the feces for neutral sterols and bile acids and the effect of these potential carcinogens on germfree animals.

To study the effect of microflora, we propose to analyze feces from population groups with a range of large-bowel cancer incidence and with differing diets (such as Japanese, Seventh-Day Adventists, and U.S. whites—both males and females of various age groups) for the bile acids, neutral sterols, tryptophan metabolites, and ammonia. We will also analyze feces of patients with polyps of the large bowel or Crohn's disease (reported to have a low risk for large-bowel cancer—Turnbull, personal communication, 1972).

We also hope to find a chemical indicator which will reflect bacterial flora. β-Glucuronidase may be such a marker, in that it mirrors the degree of microbial activity. Other indicators may be deoxycholic acid and lithocholic acid—bacterial conversion products of cholic and chenodeoxycholic acids. We believe such chemical identification would be simpler and more relevant than the identification and quantification of bacteria.

Although there is no obvious connection between the carcinogens affecting the lower intestinal tract that are used in animal experiments and environmental agents responsible for inducing colon cancer in man, there is some merit in assuming a link. Thus further studies on action of colon carcinogens in an experimental setting may explain the complex sequence of events leading to colon cancer in man. Also, comparative in vitro studies with tissues and cells from experimental animals and man may yield valuable information about metabolic parameters and response to specific carcinogens.

We therefore continue to seek an animal model and an animal carcinogen that can stimulate the production of large-bowel cancer in man.

Purpose of Present Report

We hope this review of our work will encourage others concerned with the etiology of large-bowel cancer, and will help to coordinate all investigations of large-bowel cancer. In cancer research, knowledge should be disseminated as quickly as possible. The members of the scientific community must inform and enlighten each other as research progresses.

A concerted and well-organized research effort by all investigators is important. A special study of large-bowel cancer (such as the one at the M.D. Anderson Hospital and Tumor Institute under a grant from the National Cancer Institute) is a step in the right direction. This study should lead to better coordination, planning, and enlargement of research activities in large-bowel carcinogenesis.

We report on our research in the spirit of cooperation. We have presented the epidemiologic leads on which our experimental and metabolic-epidemiologic studies are based. Since cancer of the large bowel is relatively uncommon in so many different populations, we may assume that it is largely man made and thus can ultimately be prevented by man.

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GUEST EDITORIAL — **by D. P. Burkitt**

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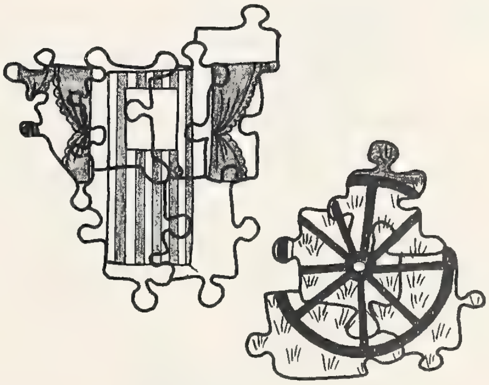
Large-Bowel Cancer: An Epidemiologic Jigsaw Puzzle

THE MANY FRAGMENTS of available knowledge regarding cancer of the large bowel lie scattered like pieces of a giant jigsaw puzzle awaiting assembly. If they could be correctly pieced together, the pattern emerging might well provide clues to the causation of this increasingly common tumor and lead to preventive measures that would save many lives and untold suffering. Epidemiologic evidence strongly suggests that bowel cancer—the second commonest cause of cancer death in the Western world after lung cancer—is very much dependent on man-made environmental factors and therefore is potentially largely preventable.

Faced with such a problem, we must relate the fragments of available evidence to one another and postulate some hypothesis for the cause of these tumors. New facts as they come to light can then be related to this hypothesis which may thereby be disproved, modified, or substantiated. Such an approach is similar to the attempted assembly of the jumbled pieces of a jigsaw puzzle. Initially, as many puzzle pieces as possible are fitted together (text-fig. 1), which corresponds to the assembly of known facts; with this as a base, imagination is now needed to formulate a hypothesis as to what the completed picture might be. The fragment of the picture so far available suggests that the whole may include a stagecoach (text-fig. 2). Some new evidence, again represented by a jigsaw fragment, may be inconsistent with the hypothesis provisionally formulated, the piece not fitting into the imagined picture (text-fig. 2). All too often the sponsor retains his particularly treasured hypothesis by sweeping the inappropriate information under the carpet, rather than respecting the evidence and, as must be done, substituting a new hypothesis both faithful to the facts already assembled and accommodating the new evidence. Thus the wheel is seen as possibly belonging to a penny-farthing bicycle and the door and windows as part of a cottage (text-fig. 3). The new piece has now found its place in the projected whole.



TEXT-FIGURE 1.—Available facts marshaled as pieces of a jigsaw puzzle assembled as far as possible.

Just such a change of interpretation of facts became necessary in the light of new evidence during my investigation into the disease which, by the generosity of former colleagues, is now known as Burkitt's lymphoma. The earlier and admittedly attractive hypothesis which visualized a vectored virus as the agent responsible for the cancer had to be abandoned in the face of evidence inconsistent with such a picture. This was replaced by an alternative concept which incriminated malaria as a cofactor, a change corresponding to replacement of the imagined carriage by the cottage and bicycle in the jigsaw puzzle. If further facts, the jigsaw pieces, continue to fit into place, the hypothesis will become a theory and the theory a fact (text-fig. 4).

THE PUZZLE PIECES

For large-bowel cancer, the box of puzzle pieces contains to date the following facts, but there are

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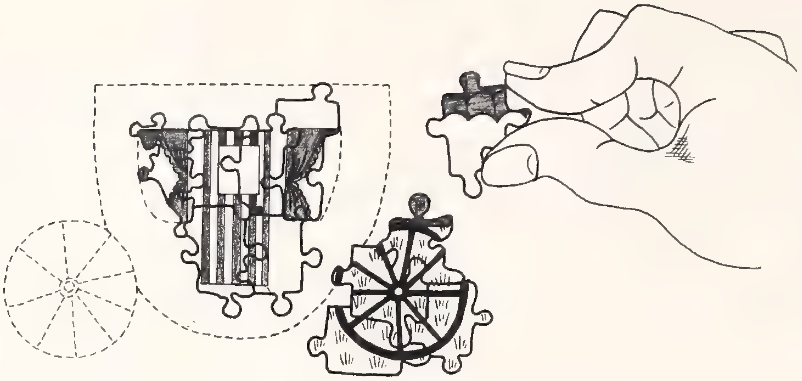
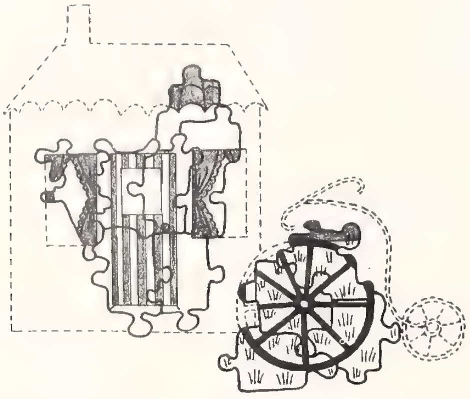
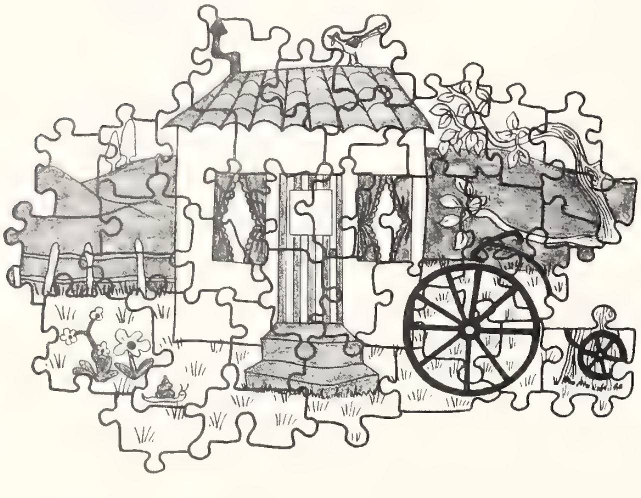
Editor's note: Periodically the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of studv The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.



Text-figure 2.—Construction of a hypothesis. A jigsaw puzzle fragment, or newly discovered fact, fails to fit.



Text-figure 3.—Erection of a new hypothesis that accommodates the new fact.



Text-figure 4.—Completing the puzzle or converting hypothesis into fact.

doubtless many other pieces as yet undiscovered. If these available pieces could be meaningfully assembled, a hypothesis could be formulated that should make it easier to search for the still missing fragments.

1) There is a close epidemiologic association between cancer of the colon and of the rectum. Both tumors have their maximum and their minimum incidence in the same populations. Variations in incidence of cancer of the colon and rectum (combined) among communities in different parts of the world are nearly four times greater than the variations in the proportion at which either site is affected relative to the other (*1*). This suggests some environmental factor(s) common to tumors in both sites, with modifying factors accounting for changes in relative proportions in different areas.

2) Cancer and adenomatous polyps are closely associated *a*) in their geographic distribution (*2*); *b*) in their anatomic distribution (*3*); *c*) in individual patients (*4*); *d*) in age distribution of the patients (*3*); and *e*) in their experimental induction (*5*). These observations suggest some causative factor common to both the benign and the malignant lesions.

3) Although the mucosal area in the small bowel is over 100 times greater than that of the large bowel, epithelial tumors are much more than 100 times commoner in the large bowel; thus, relative to surface area, the large-bowel mucosa is more than 10,000 times more liable to develop cancer, and probably in Western countries over 100,000 times more liable to grow adenomatous polyps, than is that of the small intestine. This may be partly explained by a greater susceptibility of the large-bowel mucosa to noxious elements in the fecal stream, but it suggests that the carcinogenic agent is either formed or activated in the large bowel rather than ingested in an active form in the food.

4) Anatomically, most of both the benign and the malignant tumors occur in the areas of the bowel where the feces tend to stagnate, at the beginning of the ascending colon and in the distal colon and rectum. This suggests a relationship between tumor induction and prolonged contact between the feces and the bowel mucosa.

5) The prevalence of tumors of the large bowel is closely associated with economic development (*2*) and with urban as contrasted with rural life (*6*). This suggests that both Western and, in particular, urban influences increase the carcinogenic factor that presumably resides in the feces.

6) The prevalence increases on migration from a less-westernized to a more-westernized country (*7*). This emphasizes the environmental dependence which is probably diet related.

7) Some 40 or more years ago the prevalence in U.S. Negroes was significantly less than that in U.S.

Caucasians (*8*), a discrepancy which has since disappeared. This suggests that changes in habits of Negroes over the past 40 years have increased the potentially carcinogenic factor in their feces more than have any changes in the customs of Caucasians over the same period.

8) The mucosa of the large bowel is more likely to be the site of multiple tumors than is any other body structure except the skin. In Caucasians, all of the skin surface excessively exposed to sunlight is liable to cancerous change and tumors are consequently multiple. The tendency to multiplicity of bowel tumors is consistent with a hypothesis of exposure of the whole mucosa to potentially carcinogenic stimuli.

9) Carcinogens that induce tumors in normal rats fail to do so when the rats are maintained in a sterile environment (*9*). This indicates that bacteria play a vital role in the carcinogenesis.

10) The feces from populations with a high risk of bowel cancer contain more anaerobes, particularly of the bacteroides group, than do the feces from people of low-risk areas. The bacteroides are active in degrading the bile acid cholate to deoxycholate (*10*, *11*). This further suggests that fecal bacteria play an important role in human bowel cancer and that the bacterial degradation of the bile acid cholate to potentially carcinogenic deoxycholate may be particularly significant. It is not yet certain whether deoxycholate is carcinogenic or whether it merely acts as an index of bacterial activity producing other potentially carcinogenic agents.

11) The addition to diet of fiber in the form of bran inhibits the bacterial degradation of cholate (*12*). This would indicate that the removal of cereal fiber from diet enhances bacterial degradation of cholate and therefore possibly also of other fecal constituents.

12) It has been suggested that fat alters fecal bacteria (*13*). It is known that changes in dietary fiber do this, as does intestinal obstruction.

13) Large-bowel cancer is largely a disease of later life, with an age distribution among patients similar to that of diverticular disease and hiatus hernia. This is consistent with a hypothesis that these diseases result from low-intensity or oft-repeated stimuli over a long period.

14) A number of diseases are more or less closely associated epidemiologically with large-bowel tumors, both in geographic distribution and in chronologic time of emergence as clinical problems. These include diverticular disease of the colon, cholesterol gallstones, ischemic heart disease, and hiatus hernia. Appendicitis is related, in that its prevalence has risen significantly in all communities examined several decades before any significant rise in the prevalence of the other diseases listed above (*14–17*). A good correlation has been shown between the incidence of colon cancer and of ischemic heart disease in different countries (*18*). These observations suggest that some common factor(s) has a causative function in each of these diseases.

15) Populations with relatively short intestinal transit times and with large soft stools containing low concentrations of degraded bile acids have a low prevalence of large-bowel tumors and their associated diseases. One apparent exception with regard to transit times has been reported from Hawaii, but neither the weight nor consistency of the stools was recorded (*19*). On the other hand, communities with prolonged transit times, small firm stools, and high fecal concentrations of degraded bile acids have a higher prevalence of large-bowel tumors and their associated diseases. This suggests that the factor partly responsible for these diseases acts by influencing the behavior of the gastrointestinal tract, slowing the fecal stream, producing small firm stools, and increasing bile acid degradation.

16) Intestinal transit times and stool weights and consistency are directly related to the fiber content of the diet (*20*).

17) Vegetarians in North America tend to have a lower risk of developing bowel cancer or ischemic heart disease than do nonvegetarians (*21*). This would be consistent with a causative function of meat or fat or a protective role of plant foods.

FORMULATING A HYPOTHESIS OR ASSEMBLING THE JIGSAW PUZZLE

The evidence outlined above would appear to suggest that large-bowel tumors are related to some factors characteristic of modern Western society, which slow down intestinal transit times, produce small firm stools, and alter the fecal bacterial flora. The role of these factors in the diseases associated with bowel cancer must also be postulated.

One such factor could be the lower consumption of dietary fiber, due mainly to the greater refining of flour and sugar, and also to the reduced intake of potatoes and legumes. The fiber in fruits and green vegetables is less effective, since it is more diluted and large quantities of those foods are apparently necessary if they are to compensate for these changes.

Not only are fiber-depleted diets known to slow intestinal transit times and reduce stool weights, but also they have a profound effect on cholesterol and bile acid metabolism; this has been postulated to be important in the pathogenesis of both cholesterol gallstones (*22*) and ischemic heart disease (*23*).

Whether or not changes in bacterial flora derive from changes in dietary fat or dietary fiber, the retarded bowel function associated with fiber-depleted diets provides additional time for *a*) bacterial proliferation and *b*) degradation by bacteria of bile acids.

Moreover, since addition of fiber appears to suppress bacterial degradation of bile acids (*24*), its removal must be assumed to accelerate the process.

Thus formation of more potential carcinogens may be expected with low-fiber diets, and any carcinogens so formed will be held for a prolonged time against the bowel mucosa, concentrated in a small fecal mass.

It is known that the pattern of fecal bacteria is related both to diet and to prevalence of bowel cancer.

It is known that certain anaerobes more prevalent in stools of Western populations can convert bile acids into deoxycholate and lithocholate, and glucuronide conjugates into cuprastanol and cuprastanone.

It is also known that the bacterial degradation of cholate to deoxycholate is suppressed by the addition of cereal fiber to the diet and is therefore presumably enhanced by the removal of such fiber.

It is known that fiber-depleted diets prolong intestinal transit times and consequently provide more time for bacterial activity and for the action on the bowel mucosa of any carcinogens present in the feces. It is also known that such diets concentrate fecal constituents, including any potential carcinogens, and thus presumably enhance their action.

It is not known that changes in diet alter the fecal bacteria, and there is some uncertainty as to what dietary components influence bile secretion.

The association between bowel tumors and diverticular disease could be accounted for by the fact that the fecal stasis associated with low-fiber diets results in raised intraluminal pressures that force the mucosa out between the muscle bundles in the bowel wall (*25*, *26*). The association with hiatus hernia is consistent with the only hypothesis of causation fitting the epidemiologic evidence; i.e., straining at constipated stoools may be the fundamental cause of oft-repeated intra-abdominal pressure forcing the stomach upward through the esophageal hiatus (*16*).

The consumption of fat or other postulated agents such as beef could, with fiber depletion, be cofactors, but these fail to account for the associations between large-bowel cancer and some other characteristically Western diseases.

PRACTICAL CONSIDERATIONS

The restoration of dietary fiber in the form of whole-meal bread (5 oz contains 2 g) and the addition of cereal fiber in the form of bran (2 dessertspoonsful = 2 g) quickly revolutionize bowel habits. Apart from some increase in flatus in the initial stages, ill effects are nil. There is much—we do not yet know how much—to be gained by this change, and nothing to be lost, so the betting odds are good: "Heads I win and tails I don't lose."

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ADDENDUM

To establish a hypothesis, I used (as an example of collecting pieces of information) the assembly of a jigsaw puzzle. I referred to the tentative hypothesis that the Epstein-Barr virus might be implicated in the causation of Burkitt's lymphoma. Although not yet complete, numerous pieces have been fitted into this picture and, among numerous notable advances in this field, the Epstein-Barr virus has given rise to malignant tumors in primates (*1*).

With regard to colorectal cancer, more fragments have also been found and fitted. I now view the hypothesis incriminating excessive animal fat intake, which has much to commend it, not in competition with but complimentary to the hypothesis that fiber-rich diets may be protective.

The fourfold greater colon cancer risk in Copenhagen Danes (*2*) and in New Yorkers (*3*) compared with rural Finns does not negate the causative role of fat but suggests the protective role of fiber. In both comparisons, the Finns had comparable fat intakes but consumed nearly twice as much fiber (mainly of cereal origin) as did either the Danes or New Yorkers. This resulted in the passage of much larger stools. In every situation examined, the volume of stool passed has been inversely related to colon cancer risk.

Another piece of epidemiologic evidence that fits the fat-causative, fiber-protective hypothesis is the great increase in the incidence of colorectal cancer that has occurred among Jews who emigrated from Yemen and North Africa to Israel after World War II. The reduced risk in Mormons, comparable to that of vegetarian Seventh-Day Adventists seems more in keeping with a relationship to fiber than to fat.

Numerous animal experiments have indicated that, whereas fat-rich diets tend to increase cancer prevalence, fiber-rich diets can be protective (*4–6*). Not all such experiments have led researchers to this conclusion (*7*), but failure of fiber to influence the prevalence of tumors induced by injections of dimethylhydrazine to rats cannot be extrapolated to imply that fiber-rich foods fail to confer protection against cancer in men.

Dietary fiber is now understood to comprise a number of different components that vary in their activity and their relative proportions in different plants. This variety of functions with regard to different diseases is becoming increasingly understood.

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Dietary Fat and Colon Cancer [1]

THE MAJOR EPIDEMIOLOGIC CLUE for large-bowel cancer emerges from the fact that cancer of the colon is significantly more prevalent in North America and in most Western countries than in South America, Africa, and Asia (*1–4*); its incidence is higher in the first and second generation of Japanese migrants to Hawaii and California, compared to Japanese in Japan (*4–6*). These and other epidemiologic studies indicate that colon cancer is associated with environmental factors.

Another fact supporting the environmental rather than genetic dependence of colon cancer risk is the difference in the incidence between American and African blacks (*7*). Largely on the basis of geographic and socioeconomic distribution of the colon cancer, data dealing with migrant populations, and retrospective studies of U.S. and Japanese patients with large-bowel cancer, we have proposed diet as a likely candidate for implication as a factor in the disease (*4*, *8*).

IDENTIFIED ETIOLOGIC FACTORS

The question remaining to be answered is which specific dietary factor(s) or condition(s) is involved in the etiology of colon cancer. Our studies did not relate large-bowel cancer to such factors as constipation, weight, and tobacco and alcohol usage, nor to any large-bowel diseases except for ulcerative colitis and familial polyposis (*8*). Rapid intestinal transit time and fiber content of the diet have been suggested as protective factors (*9*, *10*). Burkitt (*11*) also pointed out the elevated consumption of refined carbohydrates in high-risk populations. Arguing against refined sugars as a factor is that in Argentina, where the incidence of colon cancer is relatively high, the consumption of refined carbohydrates is low (*12*). Changes in the dietary fiber (such as consumption of white flour and refined sugar) alter intestinal transit time and stool bulk and consistency (*9–11*, *13*). Since the method used by these investigators gives inconsistent answers in replicate studies, it is desirable to compare by a more refined method the intestinal transit time of an individual on a high-fiber diet with that in the same individual on a low-fiber diet. However, there is no correlation of fiber content of food and colon cancer when judged on a worldwide basis (*14*, *15*). Neither does such a correlation exist for the intake of simple sugar (*14*, *15*). Haenszel et al. (*6*) established a positive correlation between colon cancer and the intake of legumes (string beans) which are high in fiber content. Also, animal studies indicate that azoxymethane-induced tumors in the colon were little affected as the cellulose content of the diet increased (*16*). In our studies on populations with diverse dietary habits, we found that, although there was no major difference in the weight of daily stool excretion among Americans on a mixed Western diet, American vegetarians, and Chinese and Japanese in the United States, degradation of the fecal cholesterol and bile acids was much more extensive in Americans on a mixed Western diet compared to the others (*17*).

Burkitt (*9*) suggested that carcinogens produced by the action of colonic bacteria when held for a prolonged period in a concentrated form in contact with colon mucosa account for the high incidence of colon cancer. There is no support for the belief that an increased transit time results in a decreased bacterial action on the contents of the colon. Even

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Editor's note: Periodically the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

among "fast-transit-time" populations such as those in Africa, there is sufficient time for bacterially mediated conversion of substrates in the intestinal contents into potentially cocarcinogenic and/or carcinogenic compounds. The condition responsible for the degree of degradation obviously is not the transit time but the physiologic factors (e.g., nature and amount of enzyme and substrate present and pH, to cite a few) in the lumen of the colon in which the reaction takes place. Furthermore, in colon carcinogenesis we are concerned with the qualitative and quantitative aspects of carcinogens and/or cocarcinogens present in the lumen of colon, but not with the rate at which these compounds move in the gut. The significantly higher degree of constipation in women as compared to men would also not be consistent with the sex ratio of colon cancer, which is near unity. It is unlikely, therefore, that transit time of the stool is an important factor in the etiology of colon cancer.

We have proposed that dietary animal fat and/or cholesterol are important in the etiology of large-bowel cancer (*4*, *8*, *18*). A correlation exists between dietary fat and colon cancer on a country-by-country basis (*19*, *20*). The major differences between the Japanese and American diets are in the animal fat and the combined fat contents. In general, these differences relate to the relative content of fish and carbohydrates in the diet. The equally good vital statistics and medical facilities in Japan and America substantiate the reliability of the data pointing to a great difference in the incidence of colon cancer in these two countries. An important lead also comes from the fact that, as Japanese immigrate to the United States, they adjust to the American diet, the fat content of their diet increases, and their rate of colon cancer rises significantly. The rate particularly increases in the sigmoid region of the colon, whereas the rate of cancer of the lower rectum remains unchanged. A case-control study conducted among these immigrants by Haenszel et al. (*6*) showed a positive relationship of colon cancer with the intake of beef, the major source of saturated fat in the adult population of the United States. About 35–45% of our dietary fat comes from meat. It therefore needs to be determined whether a relationship of beef to colon cancer is real, and whether beef contains carcinogenic components as such or increases the incidence of colon cancer by contributing significantly to the overall fat intake of a population, a concept that we prefer.

The above data suggest an association of colon cancer to dietary animal fat and/or cholesterol, and perhaps to a specific dietary component such as beef. It is obvious, of course, that association does not necessarily mean causation, although at the same time we recognize that, in the absence of an association, causation is not likely.

SUGGESTED HYPOTHESIS

The present data raise the question of the specific function of dietary factors in the etiology of colon cancer. It is logical to consider that constituents within the lumen of the large bowel that have tumor-initiating activity as well as tumor-promoting and -accelerating activity could relate to the development of large-bowel cancer and could stem, at least in part, from dietary habits.

Important endogenous compounds are secreted into, and thus present in, the gut, or they are derived from dietary components. They include acid and neutral steroids, bile pigments, digestive enzymes, amino acids, and fatty acids, to cite a few. The composition of diet determines the above intraluminal compounds and also the quantitative aspects of the intestinal microflora, which in turn may produce carcinogenic and/or cocarcinogenic compounds from intraluminal compounds. Thus the diet may exert its effect both by altering the supply of substrates for cocarcinogen and/or carcinogen production and by changing the nature and numbers of intestinal microflora available to act on such substrates. The importance of the microflora in general and anaerobic bacteria in particular and of bacterially modified intraluminal compounds in colon carcinogenesis is supported by the fact that the incidence of cancer of the small intestine is low. This part of the gut has a low total bacterial count and a low total anaerobic count, compared to those of the large intestine.

The most important question arises: What diet-dependent compounds, modified by intestinal bacteria, are carcinogenic and/or cocarcinogenic to colon tissue? Some investigators have become interested in the potential carcinogenic activity of certain bile acids and cholesterol metabolites because of their overall structural and steric similarity to carcinogenic polycyclic hydrocarbons. Some investigators (*21–23*) have shown that several bile acids could induce sarcomas, but, significantly, not carcinomas at the site of injection in experimental animals. In our laboratory, we instilled intrarectally in rats lithocholic acid or taurodeoxycholic acid in peanut oil after an initiating dose of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (*24*). Taurodeoxycholic acid given intrarectally is deconjugated into deoxycholic acid by microbial enzymatic hydrolysis in the large intestine. The development of adenomas significantly increased among those rats given initiating doses of MNNG and receiving bile acids as promotors compared to the group given only the MNNG, though bile acids themselves produced no tumors (*24*). Thus, in this animal model, these bile acids, present in high concentration in human stools, act as tumor promotors to an established large-bowel animal carcinogen. Nigro et al. (*25*) and Chomchai et al. (*26*) observed that the carcinogenic effect of azoxymethane in rats was enhanced by the increase of bile salts in the colon induced either by feeding cholestyramine or by diverting bile to the middle of the small intestine. Our recent in vitro studies reveal that cholestyramine-bound primary bile acids were modified extensively to secondary bile acids by intestinal bacteria (unpublished observations, 1974). The results of another experiment in which we studied the role of intestinal microflora on colon carcinogenesis indicate that germfree rats are less sensitive than conventional rats to the carcinogenicity of 1,2-dimethylhydrazine in the colon (*27*). Our recent studies also show that rats fed a high-fat diet are more susceptible to colon tumor induction by 1,2-dimethylhydrazine compared to rats fed a low-fat diet (*28*). We visualize that qualitative and quantitative differences in intraluminal compounds such as bile acids and cholesterol metabolites play a modifying role in large-bowel carcinogenesis. These compounds may stem from dietary factors, directly or indirectly via metabolism, and subsequently, are modified by the composition of intestinal microflora.

METABOLIC EPIDEMIOLOGY

In relation to the above hypothesis, Hill et al. (*29*) observed a correlation of death rate due to colon cancer in various populations to the fecal excretion and the intestinal bacterial degradation of cholesterol and bile acid metabolites. Reddy and Wynder (*17*) similarly found that the populations on a mixed Western diet, among whom the rate of large-bowel cancer is high, degraded and excreted cholesterol and bile acid metabolites to a greater degree than those populations among whom the rate of colon cancer is comparatively low. We explain this difference in bile acid and neutral sterol excretion and increased microbial modification of these compounds which obviously relate to dietary composition, mainly by a high content of animal fat and animal protein in the diet. In searching for an indicator or a diet-connected marker that may be useful in the uncovering of the causative factors of colon cancer in man and in the biochemical identification of a high-risk population, we found a positive correlation between fecal bacterial β-glucuronidase activity and excretion of microbially modified cholesterol and bile acid metabolites (*17*).

Parallel studies in vitro indicate that cholic acid and deoxycholic acid were much more extensively degraded to various microbial products by anaerobes isolated from Americans on a mixed Western diet than from American vegetarians (*30*). These data support the concept that different diets lead to typical microflora populations which control the metabolism of intraluminal compounds. One or a combination of the active strains of anaerobes, acting simultaneously or sequentially on cholesterol or bile acid metabolites or degradation products, possibly could form a cocarcinogen and/or a carcinogen.

To definitely avoid the possible genetic implications inherent in comparing various populations, we investigated the effect on the composition of fecal microflora and bile acids of a high-meat, high-fat mixed Western diet and a nonmeat diet in the same individuals (*31*). Fecal excretion of microbially modified bile acid and cholesterol metabolites and the activity of fecal microbial β-glucuronidase, which reflects microbial activity, were decreased when subjects on a high-fat, high-meat mixed Western diet transferred for 4 weeks to a nonmeat diet (*31*, *32*). The total anaerobic microflora was also higher during the period of mixed Western diet compared to that of the nonmeat diet (*31*).

Hill (*33*) noted that colon cancer patients, as compared to a control population, had the combination of a high bile acid concentration and relatively high number of the *Clostridia*, which are able to desaturate bile acids. Our preliminary data also show that patients with colon cancer and ulcerative colitis excrete high quantities of microbially modified acid and neutral sterols (unpublished observations, 1974).

All these factors (anaerobic bacteria, bile acids, and neutral sterols) indeed relate to the dietary intake of fat and/or cholesterol. Since the intestinal microflora generally is more active and some types of bacteria predominate in populations on a high-fat mixed Western diet, these reactions—including the production and release of carcinogens and/or cocarcinogens as well as other toxic components—would be more likely to occur in the colon of the populations on a mixed Western diet.

FUTURE STUDIES

Future studies ought to concentrate not only on obtaining further information on differences in the intestinal microflora and key intraluminal compounds in groups of populations on different diets as well as in patients with colon cancer, polyps, familial polyposis, and ulcerative colitis, but also on identifying chemical and/or microbial indicators which will reflect the above parameters. We believe such specific indicators would be simpler and more relevant than time-consuming, expensive, and perhaps unnecessary identification and quantification of an entire spectrum of bacteria and intraluminal compounds. Our data on β-glucuronidase (*17*, *18*) and Hill's (*33*) data on *Clostridia* and bile acids also call attention to the possibility of establishing a set of specific indicators typical of high-risk groups.

No apparent relation has been observed so far between diet-mediated agents responsible for colon cancer in man and the carcinogens affecting the large intestine in animals. There is, however, some merit in assuming a link, because experimental studies in animal models may explain the complex sequence of events leading to colon cancer in man. It is of major importance, however, to select animal models based on the composition of intraluminal compounds, including intestinal bacteria, and on the ability of these animals to adjust to different types of diets.

CONCLUDING REMARKS

Although a specific carcinogen for colon cancer has not yet been identified in the feces, an association has been established linking colon cancer to dietary fat and/or cholesterol but not, in our opinion, to other factors. In the animal model, two bile acids (deoxycholic and lithocholic acid) have been shown to be tumor promotors in the colon. The association of the consumption of beef to colon cancer among Japanese immigrants to the United States may be explained in that beef contributes 35–45% of the total fat content of the American diet. We do not believe that transit-time of the intestinal contents is an important factor, because whatever bacterial conversion takes place in the stool components has already done so in the relatively rapid transit time reported to occur among Africans.

It has been proposed that the tumorigenic activity of the large-bowel contents is linked to fecal anaerobic bacteria and substrates such as acid and neutral sterols. Although we still need to understand the specifics of these relationships to colon carcinogenesis, it appears that the dietary fat and/or cholesterol concept is basic to any subsequent development of carcinogens and/or cocarcinogens and promotors, all of which may have a function in the pathogenesis of colon cancer. In the meantime, it has to be recognized that historically the final elucidation of the mechanisms of carcinogenesis has not been necessary to reduce their carcinogenic effect. Therefore, it is important that we consider the managerial approach to preventive medicine. We suggest that the "Prudent Diet" (*34*), believed to be so important for the reduction of cardiovascular disease for a largely sedentary population, may be recommended for its possible beneficial effect toward a reduction of colon cancer and perhaps of other types of diet-related cancers.

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Lung Cancer: A Status Report

Bronchogenic carcinoma presents as four relatively distinct diseases: epidermoid carcinoma, small-cell carcinoma, adenocarcinoma, and large-cell anaplastic carcinoma. Differences in the biologic behavior of these cell types account for the differences in mode of presentation (*1*), in tendency toward early metastases (*2*), and in responses to therapy (*3*). In this review, the natural history of a hypothetic lung cancer patient will be discussed, starting with attempts at early diagnosis through treatment with the use of the currently available therapeutic modalities. Throughout the discussion, gaps in our knowledge will be emphasized.

Most patients with newly diagnosed lung cancer seek medical attention because of the onset of disease-related symptoms (*4*). The outlook for such patients is poor, with overall 5-year survival rates of about 5–7% (*5*). Early detection studies have attempted to improve this prognosis; they have been of two types: mass roentgenographic screening at 6-month intervals and serial sputum cytology studies at similar intervals. High-risk individuals defined as "cigarette-smoking males over age 40" comprise the study population. The mass roentgenographic screening programs have largely failed. With the use of the Philadelphia Pulmonary Neoplasm Research Project as an example, the overall 5-year survival rate for 94 patients with histologically diagnosed cancer was 6%. Only 37% of the 94 cancers were resectable at diagnosis and even in this group the 5-year survival rate was only 18% (*6, 7*). Similar results in the detection of asymptomatic individuals by mass radiography have been reported by authors from several European countries (*8–10*). Radiographic studies have limitations accounting for the observed results. 1) Lesions generally have to be about 1 cm in diameter to be seen (*3*). Such lesions are already advanced and represent about 30 doublings of a hypothetic single tumor cell in a natural history of disease that may encompass approximately 40 doublings (*11*). 2) There are frequent difficulties in the roentgenographic detection of malignant lesions. They may be obscured by preexisting X-ray abnormalities of chronic pulmonary disease or by normal structures seen on X-ray, e.g., the ribs or heart shadow (*12*).

A more promising approach to early diagnosis is the serial collection of sputum samples for cytologic examination. The basis for this approach is the observation that lung cancer patients shed cells demonstrating increasing degrees of cytologic atypia for a long time before the detection of evident malignancy (*13*). Although an early collaborative American Cancer Society–Veterans Administration study used a single sputum sample for each time interval (*14*), more recent work showed that the detection rate increases from 45% with a single sample, to 86% for three sputums, and to 95% when five sputums are evaluated (*3*). After cytologic detection in the X-ray negative, individual tumors may be localized by fiberoptic bronchoscopy (*15*), bronchography (*16*), or detection of regional ventilation-perfusion abnormalities with ^{133}xenon radiospirometry (*17*). Although the numbers of patients with cancer detected by cytology is still small, investigations at three institutions indicate that resectability rates in such individuals will exceed the 35–60% resectability rates of patients detected in radiographic screening studies (*18–20*).

Early detection studies would be advanced if one could more narrowly define the population at high risk of developing lung cancer. One possible lead is the observation that patients with bronchogenic carcinoma had higher levels of inducible aryl hydrocarbon hydroxylase (AHH) in their peripheral blood lymphocytes (*21*) and monocytes (*22*) than did suitable control groups, and also that smokers had higher levels of AHH in their pulmonary alveolar macrophages than did nonsmokers (*23*). Since the role of this enzyme as either a protector against, or as a promoter of, pulmonary carcinogenesis is not established, and since AHH levels have not been directly measured in the bronchial mucosa, further studies are clearly needed. A second line of investigation questioned whether α-1 antitrypsin deficiency predisposed a person to lung cancer. Both homozygous and heterozygous deficiency of this enzyme is associated with pulmonary emphysema, though in heterozygous individuals it is only manifest in those who smoke (*24*). A study on 73 patients with nonresectable histologically diagnosed lung cancer showed elevated α-1 antitrypsin levels (*25*).

At present, therefore, sputum cytology must be relied on for early diagnosis. A logical question then would be whether cytologic examination alone is sufficient to determine histologic cell type. A 90–100% concordance between cytologic diagnosis and subsequent histologic diagnosis has been established for well-differentiated and moderately well-differentiated epidermoid carcinoma, adenocarcinoma, and small-cell carcinoma [(*26*) and Kanhouwa S, Matthews MJ: Manuscript in preparation]. For individuals with less-differentiated tumors or in those who have negative sputum cytologies, other diagnostic techniques must be used, including fiberoptic

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Editor's note: Periodically the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

bronchoscopy with biopsy of either bronchial mucosa (*15*) or pulmonary parenchyma (*27*) and transthoracic needle biopsies and/or aspirates of lung or pleura (*28, 29*). With present methods, fewer patients should require thoracotomy, mediastinoscopy, or other surgical exploration for diagnostic purposes.

With the establishment of a cytologic or histologic diagnosis, the next step is the staging of disease, the primary purpose of which, for all cell types except small-cell carcinoma (*30*), is the determination of tumor resectability. Methods for the evaluation of intrathoracic involvement by bronchogenic carcinoma are numerous and can be divided into angiographic studies, i.e., superior vena cavography (*31*), azygous venography (*32*), and pulmonary angiography (*33*), and surgical procedures, i.e., scalene node biopsy (*34*), mediastinoscopy (*35, 36*), and mediastinotomy (*37*). To determine the relative merits of these techniques, information is needed on the number of patients with resectable cancer before study, the number of patients considered inoperable on the basis of the study, and finally the percentage of resectability and the survival of patients with negative studies, with information on the site of first relapse in patients who die. One would also like a breakdown of the above categories by histologic cell type, since mediastinal involvement is present on initial evaluation in about 70% of patients with small-cell carcinoma versus about 16% for those with well-differentiated epidermoid carcinoma (*38*). The three chains of lymph nodes in the superior mediastinum must also be considered: an anterior (prevascular), a posterior (esophageal), and a paratracheal chain. The last is best evaluated by mediastinoscopy and represents the primary drainage chain for all lung areas except for the left upper lobe and left hilum. These two lung areas are difficult to evaluate by angiographic techniques because of the paucity of vessels in the area (*32*); they are also difficult for the surgeon doing mediastinoscopy, since drainage is to the anterior nodes frequently hidden by the aortic arch (*39*). Left upper lobe and left hilum lesions may thus be best evaluated by mediastinotomy (*37*). Both the angiographic procedures and mediastinoscopy will establish nonresectability of disease in about one-third of patients clinically thought to be operable. There are less data in the literature on false-negative rates, though in one study combining pulmonary angiography and azygous venography there were about 50% false negatives (*32*). The false negativity of mediastinoscopy is in the area of 10–30%, with the higher percentages in left lung lesions (*39, 40*). The combination of pulmonary angiography and mediastinoscopy may have some benefit, since a report indicates that when both were negative, the resectability rate was 97% (*41*). This is not unexpected, because pulmonary angiography allows the evaluation of the main pulmonary vessels, pericardium, right atrium, innominate vein, and superior vena cava (*42*), areas not seen well on mediastinoscopy.

Staging for disease outside the thorax has been reviewed by Muggia and Chervu (*43*) and only certain points need to be emphasized. The frequency of distant metastases is related to histologic cell type, such that in a study of patients who died of causes unrelated to cancer within 1 month of what was thought to be a "curative" resection, distant metastases were present in 22 of 131 patients with epidermoid carcinoma (17%) versus 12 of 19 with small-cell carcinoma (63%) and 12 of 30 with adenocarcinoma (40%) (*2*). Metastases were present in so wide a variety of organs as to make work-up by specific organ sites impractical. What appears necessary for more accurate staging is the development of tumor-seeking radionuclides that will concentrate in tumor tissue, and improvements in present day scanning technology so that smaller lesions may be seen. Recent interest has focused on two radiopharmaceuticals—^{67}gallium citrate (*44*) and ^{111}indium bleomycin (*45*). Thus far, neither is sufficiently reliable for routine detection of disease at nonclinically apparent sites. Other techniques, i.e., diagnostic ultrasound (*46*) and the newly developed computerized X-ray tomography of the brain (EMI scan) (*47*) or the whole-body (ACTA scan) (*48*) may be of future help.

Completion of the staging work-up allows a consideration of the various therapeutic modalities. For nearly all patients with bronchogenic carcinoma, surgery represents the only chance for cure. Surgical resectability depends on two factors: the physiologic status of the host and the stage of disease. Physiologic evaluation of operability involves an assessment of whether a patient can survive the anticipated surgery. Although attention is given to cardiopulmonary reserve, other physiologic parameters including renal and liver function, and general physical condition are also considered. The evaluation of pulmonary function has included standard pulmonary function tests, arterial blood gases, and ventilation-perfusion studies of the contralateral normal lung (*49*). At present, it appears useful to combine two or more of the above parameters, as has been done by Olsen et al. (*50*) and Lindell (*51*).

Staging is frequently recorded in the tumor–node–metastases (TNM) system. Factors of importance with regard to the primary tumor (T) include the diameter of the lesion, the presence or absence of associated atelectasis or pneumonitis, the most proximal extent of the tumor on bronchoscopy, the presence of pleural effusion, and if there is local extension of tumor to adjacent structures. For regional nodal involvement (N), the first nodal barrier is the hilar lymph nodes, with mediastinal nodes and more distal nodes involved subsequently. For distant metastates (M), only presence or absence is important. A simplified version of the TNM system and clinical stages is shown in table 1. For more detailed description, see the work of Carr and Mountain (*52*). The TNM stage correlates with prognosis for all histologic cell types except small-cell carcinoma. In this cell type, the 5-year survival of all clinical stages is less than the expected mortality for surgery, and thus most surgeons believe that the diagnosis of small-cell carcinoma is a contraindication to excisional surgery (*5*). Surgeons agree that patients with stage I and stage II epidermoid carcinoma, adenocarcinoma, and large-cell anaplastic carcinoma should have surgery if it is physiologically feasible (*53*). The surgeon should sacrifice as small an amount of lung parenchyma as possible, consistent with complete tumor resection. Thus when feasible, lobectomy is preferred to pneumonectomy, and, on occasion, wedge resections and sleeve resections with appropriate tracheobronchial reconstruction may be done (*53–55*). The reason for lesser operations than pneumonectomy is a lowered surgical mortality from about 12–15% for pneumonectomy to about 5% for lobectomy without a sacrifice in 5-year survival in appropriately staged individuals (*56, 57*). Of greater controversy is the surgical management of stage III disease on the basis of ipsilateral mediastinal adenopathy, contralateral involvement being a contraindication to surgery. A possible favorable situation for surgery in such a patient would be the solely intranodal involvement of a single ipsilateral mediastinal node, located below or near the carina (*53*) in a patient with epidermoid carcinoma (*39*). Any extension of tumor outside the nodal capsule and involvement of nodes high in the mediastinum make it unlikely that the 5-year survival rate would exceed the expected surgical mortality. Along a similar line, there is little indication for palliative resection of tumor. Thus in a Veterans Administration Surgical Adjuvant Group study of 243 patients with palliative resection, 55% were dead within 6 months and 74% by 1 year. Only 9 (4%) survived 5 years and all had epidermoid carcinoma thought to have been completely resected, with only the pathologist detecting microscopic tumor in the margin of the resected specimen (*58*). All patients with known residual mediastinal disease were dead in 3 years, with only 16% surviving 1 year (*58*). Other factors influencing survival in patients with resected cancer have been documented by Shields and Robinette (*59*). Favorable factors included low disease stage, age less than 60 years, and involvement of the right lung. Unfavorable factors related to weight loss, preexisting pulmonary disease, necessity for pneumonectomy, and blood vessel or lymphatic involvement or microscopic examination.

Another subset of patients to be considered regarding surgical therapy is those with tumors, usually either well-differentiated or moderately well-differentiated epidermoid carcinomas, that directly extend into the chest wall and ribs. Such tumors in the superior sulcus give the characteristic findings of arm pain in an ulnar distribution, Horner's syndrome, and rib erosion (*60*). These tumors generally do not involve hilar or mediastinal nodes, and good results have been obtained with preoperative irradiation followed by en bloc pulmonary and chest wall resection (*61*).

Surgical adjuvant studies involving either preoperative or postoperative radiation therapy or chemotherapy will be covered in the specific modality discussion.

Radiation therapy, like surgery, represents localized treatment for localized disease and thus requires accurate staging if used as curative. Also, like surgery, the physiologic status of the host must be considered in treatment planning, since radiation pneumonitis and progressive pulmonary fibrosis invariably accompany current radiation therapy prescriptions (*62*). These and other complications of radiation therapy will be considered subsequently.

The principal question to be asked about radiation therapy is whether any currently used radiation doses and schedules can sterilize the tumor within the treatment field. Histologic material for an answer to this question is available from preoperative radiation studies and autopsy studies. In four preoperative studies which included 250 evaluable patients treated with megavoltage radiation doses of 4,000–6,000 rads, local control of disease was achieved in 27–40% (*63–66*). Unfortunately, those achieving local control had no better survival than did the '"noncontrolled" group. Three autopsy studies involving 101 patients who received 4,000–6,000 rads demonstrated local control in 20–38% (*67–69*), also without survival benefit. Poor survival results in patients attaining local control results from several factors including the presence, at radiation therapy, of subclinical systemic metastases, and the possibility that thoracic irradiation is immunosuppressive and favors development and proliferation of distant disease (*70*). The immunosuppression question has been addressed in limited fashion in lung cancer; only one study showed an increased incidence of systemic metastases in irradiated patients (*71*) and two other investigations showed no difference in metastatic patterns of irradiated patients versus controls (*67, 69*). Finally, survival in irradiated

TABLE 1.—*TNM classification and staging*

Primary tumor (T)					
Category	Tumor diameter	Atelectasis and/or obstructive pneumonitis	Bronchoscopy finding: most proximal tumor extension	Extra pulmonary involvement	
				Pleural effusion	Local extension: chest wall, mediastinum
T1	≤3 cm	None	Lobar bronchus	None	None
T2	>3 cm	<Entire lung	≥2 cm from carina	None	None
T3	Any size	Entire lung	<2 cm from carina	Present or absent	Present or absent
			Regional nodes (N)		
			Ipsilateral hilar		Mediastinal
N0			None		None
N1			Present		None
N2			Present or absent		Present
			Metastases (M)		
M0		No metastases			
M1		Extrathoracic or contralateral thoracic metastases			
			Stage		
	I		II		III
	T1 N0 M0		T2 N1 M0		T3 any N or M
	T1 N1 M0				N2 any T or M
	T2 N0 M0				M1 any T or N

patients may be adversely affected by complications and toxicity inherent in the treatment. Acute radiation complications seen with preoperative therapy include delayed wound healing and increased incidence of intrathoracic infections (*63, 64*). More chronic and possibly more serious complications include transverse myelitis and pulmonary fibrosis, both of which are mediated by a radiation-induced vasculitis with resultant vascular occlusion. Radiation myelitis is estimated to develop in 1–5% of patients treated with megavoltage radiation of 5,000 rads in 5 weeks who are followed for 5 years (*72*). Myelitis most often occurs 12–24 months after radiation and is rare before 6 months. The upper thoracic cord is most susceptible to this complication due to a poor blood supply relative to the rest of the spinal cord (*73*).

Radiation pneumonitis and fibrosis are the most significant complications of therapy. After treatment with 4,000 rads in 4 weeks, pneumonitis can be evidenced within 8 weeks, and generally pneumonitis occurs 1 week earlier for each additional 1,000 rads of delivered radiation (*62*). All patients developing pneumonitis go on to develop fibrosis which usually stabilizes by 9–12 months post therapy (*72*). As a rule, X-ray changes associated with radiation pneumonitis can be correlated with changes in pulmonary function tests, especially in measurements of static lung volumes. However, pulmonary function may improve in irradiated patients because of the correction of regional ventilatory and perfusion abnormalities caused by tumor (*74*). The observation that patients with severe chronic bronchitis can withstand radical radiotherapy to the lung further indicates that preexisting abnormalities of pulmonary function are not an absolute contraindication of radiation therapy (*75–78*).

Curative radiotherapy is a distant goal, but we now know several things that may aid future efforts. The minimal dose required for the sterilization of disease apparently is above 4,000 rads (*67–69*). Further, it seems that various split-course techniques may give improved results over continuous radiation therapy (*79, 80*). A fault of most of the above trials is that patient survival rather than local sterilization was the parameter studied. The autopsy study of Rissanen et al. (*67*) on split-course versus continuous radiation therapy was a notable exception. They demonstrated that 78% of all patients achieving local control were treated by the split-course method.

The major value of radiation therapy at the present time is the palliation of distressing symptoms such as pain, cough, hemoptysis, neurologic symptoms, and superior vena caval and bronchial obstructions. Palliation can be achieved in 75% of patients with smaller doses of radiation therapy than are used for curative attempts (*72*).

Future developments in radiation therapy will probably include better dose fractionation schemes and also the testing, on a wider scale, of high linear energy transfer (LET) particles such as neutrons, pimesons, and stripped nuclei. This type of radiation may destroy hypoxic cells more effectively than low LET radiation or may give better distribution of radiation energy (*72*).

CHEMOTHERAPY

Numerous chemotherapy trials have been done on lung cancer patients with either single drugs or two or more drugs combined. These studies have been reviewed by Selawry (*81–83*). Major progress has been made in the chemotherapy of small-cell carcinoma. For this cell type, combination chemotherapy is clearly superior to single agent treatment, with partial responses seen in over 50% of patients on various regimens and complete responses in 10–30% (*84–86*). Tumor response has resulted in some increase in survival, but a need remains for improved methods of remission consolidation and maintenance. For epidermoid carcinoma, adenocarcinoma, and large-cell anaplastic carcinoma, studies conducted on patients with advanced disease have given leads to improved chemotherapy, but, as yet, significant complete tumor regressions have not been achieved and only slight increases in survival are recorded. Improvement in chemotherapy requires identification of new active agents and drug combinations and also improvements in the methodology of conducting and evaluating clinical trials. Prognostic factors important in determining treatment response must be identified, so that one may determine comparability of study results of different groups, and a need for other criteria besides tumor measurements are necessary to serve as parameters of response. On a more practical level, one also needs explanations for the failures of surgical adjuvant chemotherapy and for combination radiation therapy–chemotherapy, since efficacy of chemotherapy would be expected in situations where the tumor burden is decreased. The above questions will be considered below.

Prognostic factors for stratification include histologic cell type, clinical or surgical stage of disease by the TNM classification, and performance status expressed either in the Karnofsky or Zubrod scale (*87*). Other prognostic components that might be useful for stratification include delayed hypersensitivity reactivity (*88, 89*) and the presence or absence of weight loss of 10 pounds or more (*90*). Feinstein's symptom classification (*4*) probably relates to the biologic behavior of the various cell types of bronchogenic carcinoma and need not be considered an independent prognostic variable.

Response to chemotherapy is usually discussed in terms of a greater than or equal to 50% shrinkage of tumor masses or as an improvement in patient survival. Both these parameters have inherent difficulties. In most lung cancer patients, an abnormality on chest X-ray is the only "measurable" lesion. Interpretation of such abnormalities is frequently complicated by the presence of associated atelectasis and pneumonitis (*52*), by pulmonary fibrosis resulting from previous X-ray therapy (*91*), or because of radiologic abnormalities secondary to preexisting chronic pulmonary disease (*12*). Other parameters of response are available but, unfortunately, are infrequently used. Several investigators have shown morphologic changes, demonstrable by both light and electron microscopy, in tumor cells of patients responding to therapy (*92, 93*). Such alterations might be an unequivocal index of response to treatment, and furthermore, loss of characteristic morphologic changes might be an early indication of progression on a given therapy. Thus serial examination of sputum cytologies and serial repetition of fiberoptic bronchoscopy with direct visualization and biopsy of the tumor should probably be more routine.

Survival, as a parameter of response, is not entirely reliable. One does not have to attend many postmortem examinations to realize that many patients die with, but not necessarily of, bronchogenic carcinoma. For many, the cause of death cannot be identified. Such patients are often cachectic and death is attributed to metabolic derangements. Tumor burden in such individuals may be minimal. Conceivably, the definition and treatment of factors related to development of tumor cachexia might prolong survival of such patients irrespective of any direct antitumor effect.

The results of surgical adjuvant trials and of combination radiotherapy–chemotherapy trials have been disappointing. Possible reasons for poor results of surgical adjuvant studies are the inclusion of the nonhomogeneous patient populations with a broad spectrum of prognoses (*94*), use of chemotherapeutic agents in what were probably improper schedules, either because of too short a duration of chemotherapy or of prolonged relatively frequent drug administration possibly causing an immunosuppressive effect (*95, 96*). In addition, most studies were done with a single chemotherapeutic agent (either mechlorethamine or cyclophosphamide) or two single agents (cyclophosphamide and methotrexate) given sequentially (*95*). Of some promise was a four-drug combination of cyclophosphamide, methotrexate, fluorouracil, and vinblastine. Unfortunately, this suffered from several defects listed above, including nonhomogeneous patient populations, all varieties of histologic cell types (including small-cell carcinoma in the treated but not the control group), and what appeared to be a greater degree of supportive care in the treated group (*97*).

Combined radiotherapy–chemotherapy trials were based, in part, on the rationale that chemotherapy cell kill would improve oxygenation of tumors through improved perfusion or that the chemotherapeutic agent might act as a radiosensitizer (*98, 99*). Thus far, benefits of this type of combined therapy have been minimal. Thus Bergsagel et al. (*100*) using cyclophosphamide (1 g/m^2) every 3 weeks and radiation therapy in patients with nonresectable lung cancer found little effect on the primary tumor but observed a delay of about 90–100 days in the appearance of distant metastases in the cyclophosphamide group. This delay was reflected in improved survival from 216 days in the radiation therapy group to 306 days in the combined modality group. By contrast, a European group studying radiation therapy with or without cyclophosphamide showed a greater tumor shrinkage of the primary lesion as evaluated by bronchoscopy, with no significant effect on survival (*101*). Most other combination studies did not show even these hints of activity and one must also wonder whether combinations of these two modalities might potentiate each other's immunosuppressive effects.

IMMUNOTHERAPY

Several lines of investigation support the use of immunotherapy in lung cancer patients. Thus soluble antigens with some tumor specificity have been extracted from surgically resected small-cell carcinoma and adenocarcinoma of the lung (*102*). Patients with reactivity to recall skin test antigens have a better prognosis than do patients anergic to such tests (*88, 89*). Also patients who postoperatively develop empyema, a probable nonspecific immune stimulus, seem to have a better prognosis than individuals who do not (*103*). Further, since radiotherapy (*104*) and chemotherapy (*105*) are immunosuppressive, it seems worthwhile to attempt to reverse this suppression. Thus far, most lung cancer immunotherapy studies use active nonspecific immunotherapy with organisms such as BCG or *Corynebacterium parvum* with or without chemotherapy (*105, 106*). Treatment has usually been via cutaneous inoculation, though other routes are being explored, including the direct installation into the bronchial tree (*107*) or the pleural space (*108*). Active specific immunotherapy studies with tumor antigens such as those extracted by Hollinshead et al. (*102*) are being planned. Adoptive immunotherapy trials with materials such as the transfer factor (*109*) and immunogenic RNA (*110*) may be developed. Other nonspecific immune stimulators, i.e., levamisole, methanol-extraction residue, and thymosin, are being studied. Hopefully, in the next few years significant advances will be made in these areas.

In summary, an attempt has been made to indicate where we now stand in early diagnosis, in efforts directed toward the choice of the appropriate therapeutic modality, and in various important and unanswered questions within each therapeutic modality. Answers to many of these questions appear to be within reach and may enable us to make significant progress against bronchogenic carcinoma.

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ADDENDUM

This communication updates various aspects of lung cancer biology and treatment.

Lung cancer incidence and mortality rates may be levelling off in males. However, in females, these rates are dramatically increasing, concomitant with increased cigarette consumption. Dissemination of smoking risk information remains essential in lung cancer management.

Surgery is the only curative treatment modality for most patients with lung cancer. To detect resectable patients, screening programs that use every 4-month sputum cytology plus chest X-ray examination have been established. Early results of these trials are promising. Conclusive demonstration of benefit requires longer follow-up.

Extension of early detection studies to the general population requires more accurate definition of a high-risk group for lung cancer development. Currently, this group includes cigarette-smoking males; presumably, females 45 years of age or older will soon be included, with or without various occupational, environmental, or familial risk factors. The initial optimism that elevated aryl hydrocarbon hydroxylase levels might predict lung cancer susceptibility has been tempered by recent studies. Other markers of susceptibility are necessary. Continued investigation into variability of drug and carcinogen metabolism in cancer patients and controls seems a promising approach.

Computerized tomography, a technique that may be useful in preoperative staging, has become widely available. In addition, it may aid in distinguishing benign from malignant intrathoracic lesions. An additional role is in radiotherapeutic treatment planning.

Disease stage is the principal prognostic determinant of survival. Stage is usually assigned after prethoracotomy work-up or on gross inspection at surgery. Stage is most accurately assessed, however, by pathologic examination of resected tissues, especially when mediastinal lymph nodes are extensively sampled. At Memorial Sloan-Kettering Cancer Center, actuarial 3-year disease-free survival for histologic stage I individuals was 77%. For patients with $T_1N_0M_0$ tumors, 3-year disease-free survival approximated 90%. If these results are confirmed, certain stage I patients, i.e., $T_1N_0M_0$, either may not be candidates for adjuvant trials or multi-institution cooperative groups will be needed to obtain sufficient patient entry.

Various immunotherapeutic regimens may be useful surgical adjuvants. Current results have recently been summarized in (*1*).

Radiation therapy advances include the development of new techniques such as hemibody radiation therapy and the adjunctive use of radiation sensitizers and hyperthermia.

Chemotherapy progress is most evident in small cell carcinoma. Reports of long-duration, disease-free survivors are appearing; with prolonged survival, new clinical problems are emerging. These include the frequent occurrence of central nervous system metastases and the enhanced local and systemic toxicities observed with combined chemotherapy–radiation therapy regimens.

The prevailing mood in lung cancer research is cautious optimism. Two international conferences in the past 2 years, the Second National Cancer Conference on Lung Cancer Treatment and the International Association for the Study of Lung Cancer World Conference on Lung Cancer have stimulated numerous ongoing clinical and laboratory investigations.

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EDITORIAL

by Stephen K. Carter, M.D.[1]

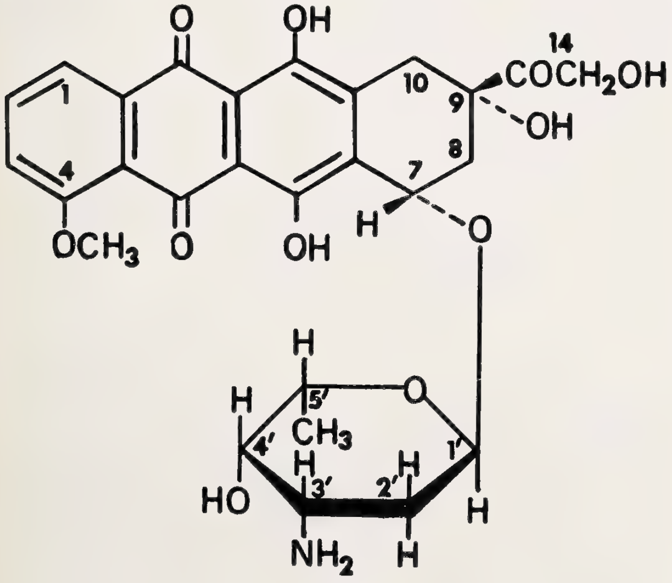
JNCI 55:1265–1274, 1975

Adriamycin—A Review

Cytotoxic chemotherapy as a modality in modern cancer therapy began about 30 years ago with the discovery of the clinical activity of nitrogen mustard by a group at the Sloan-Kettering Memorial Hospital, New York, New York. Since then, the armamentarium of the chemotherapist has grown substantially and is one of the major factors that led to full flowering of medical oncology as a subspeciality of internal medicine. Today, more than 30 cytotoxic drugs are available with a wide range of structures, mechanisms of action, and clinical activity. These antitumor drugs come from a variety of sources (synthetic chemicals, antibiotics, plant products, enzymes) obtained in different geographic locations. Adriamycin, a new drug that recently became commercially available in the United States, is adding significantly to the effects achieved with cancer chemotherapy. It is the purpose of this editorial to put this drug into current perspective.

PRECLINICAL STUDIES

Adriamycin is an anthracycline antibiotic originally isolated by aerobic fermentation of *Streptomyces peucetius caesius* and by solvent extraction of chromatographic purification (*1*). Structurally, adriamycin is an analogue of daunorubicin, an earlier clinical compound, differing from it only in the hydroxylation of the 14th carbon (text-fig. 1). Daunorubicin has clinical activity against acute leukemia, but a narrow therapeutic index (TI) limits its usefulness. The biologic activity of adriamycin is related to its ability to bind specifically with DNA by intercalation between adjacent base pairs of the double helical structure (*2, 3*). This property, inducing stereochemical template disordering, inhibits the enzymes involved in DNA replication (DNA-dependent DNA polymerase) and transcription (DNA-dependent RNA polymerase) (*4*). These results, obtained by studies of cell-free systems, are consistent with the biologic data obtained in in vivo and cell cultures [i.e., inhibition of the incorporation of precursors into DNA and RNA, inhibition of mitosis, and induction of chromosome aberrations (5–8)].



Text-figure 1.—Structure of adriamycin.

Adriamycin was developed by the Farmitalia Company, Milan, Italy, and its choice over daunorubicin was based on data from the Ehrlich ascites tumor in mice (*9, 10*). Activity has also been observed in C3H mice with transplanted lymphosarcoma (*9*), rats bearing Oberling-Guérin-Guérin myeloma (*10*), mice bearing sarcoma 180 (*9*), and in leukemia L1210 (*11*), the major screening tool in the drug development program of the Division of Cancer Treatment (DCT). Activity in other systems was also described (*12–14*).

At the cell level, the drug has an immediate and dose-related inhibitory effect on mitosis (*5*). At low doses, the inhibition is at the preprophase stage, whereas at higher doses the mitotic block is complete. Adriamycin induces chromosome aberration in human leukocyte cultures (*15*). The extent and type of chromosome aberration is dose related, with increasing frequency and severity as the drug dose is increased. The changes observed have been mutagenic (*8*).

Adriamycin also has immunosuppressive effects. It significantly inhibits the titer of hemolytic and hemagglutinating antibodies in mice immunized with sheep red blood cells. The greatest immunodepressant effect was recorded when adriamycin was administered after antigen administration, thus indicating that adriamycin does not act on the very early steps of the immune response (*16, 17*). Activity has also been observed in both the primary and transplanted tumors induced by the Moloney strain of murine sarcoma virus in mice (*18*) and female DBA/2J mice bearing ascites lymphocytic leukemia P-288 (*19*).

Pharmacologic studies show that adriamycin administered iv is rapidly cleared from the plasma of rodents, with concentration of the drug in the liver, spleen, kidney, lung, and heart (*20, 21*). Drug excretion is prolonged and occurs predominantly via the liver.

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

The pharmacology of adriamycin in man has been extensively studied by Benjamin et al. (*22–24*), Bachur et al. (*25*), and DiFronzo et al. (*26*). The drug has a rapid plasma clearance and a large volume of distribution, suggesting wide dispersion into the tissue (*22, 26*). There is minimal urinary excretion, with only 5% of the drug excreted during the first 5 days, as measured by fluorimetric methods; this suggests extensive tissue binding. After injection of a tritium-labeled drug at a dose of 0.5 mg/kg, 50% of the radioactivity was detected in the feces in 7 days, whereas in patients with impaired hepatic function, the radioactivity dropped to only 20% (*26*).

Adriamycin is metabolized predominantly by the liver to adriamycinol and several aglycone derivatives; approximately half the drug is excreted intact in the bile, with an additional 30% excreted as conjugates (*22, 25*). Pharmacokinetic studies in patients with hepatic dysfunction show significant, prolonged plasma levels of adriamycin; metabolites are associated with exaggerated clinical toxicity (*24*). These observations are the basis of a requirement for dose deescalation in patients with impaired hepatic function.

CLINICAL STUDIES

Adriamycin has been clinically tested in the United States since 1969, and over 3,000 patients have been treated with the drug alone or in combination with other chemotherapeutic agents.

One of the most impressive aspects of the antitumor effect of adriamycin is its broad spectrum of activity (table 1). In nine tumor categories adriamycin has established anticancer activity. These encompass a broad range of solid tumors that in the past have been relatively insensitive to chemotherapy, especially the soft tissue and bone sarcomas and bladder cancer.

In seven additional tumor categories available data indicate some degree of adriamycin activity, although a definitive statement cannot be made yet. This group also includes some classically unresponsive tumor types such as cancer of the stomach and prostate. Only three tumor categories are unresponsive to adriamycin; they include some tumors that generally are least sensitive to chemotherapy (e.g., renal cancer and malignant melanoma). Despite the extensive clinical testing of adriamycin, there are still five categories for which no adequate data exist. This will soon be rectified, since various cooperative clinical groups have nearly all these tumors under phase II evaluation with adriamycin.

When an experimental drug is active in phase II evaluations, a study approach is established involving both clinical trials and preclinical laboratories (text-fig. 2). In clinical studies, the new drug is combined with other active drugs or integrated into established combinations to increase the therapeutic activity against advanced disease. Additionally, in keeping with the overall thrust of combined modality attacks against cancer (*27*), a new active drug is combined with surgery and/or radiotherapy and/or immunotherapy to increase the remission rate for primary therapy. The current status of the flow of adriamycin into combination regimens and combined-modality approaches is outlined in table 2. Simultaneously, the following ranges of approaches are taken to improve the TI of an active drug: 1) schedule manipulation, 2) dose manipulation, 3) pharmacologic investigations, 4) attempts to block toxicity, and 5) clinical experience. Finally, a vigorous search for analogues is made to find a new drug with greater efficacy and/or diminished toxicity.

TABLE 2.—*Current status of adriamycin studies*

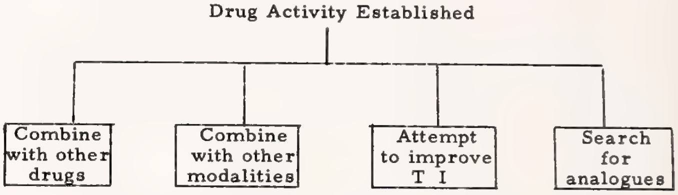
Tumors	Studies in progress [a]	
	Combination regimen superior to single agent	Combined modality role
Breast cancer	Yes[b]	Under test
Sarcomas:		
Excluding osteogenic	Yes[c]	Under test
Osteogenic	Under test	Positive
Lung cancer	No	No
Malignant lymphomas	Yes (various drugs)	Under test
Bladder cancer	No	No
Testicular cancer	No	No
Thyroid cancer	No	No
Pediatric tumors	Under test	Under test
Acute leukemias	Yes	No

[a] Single-agent activity studies are being conducted for all tumors listed.

[b] Adriamycin + cyclophosphamide; adriamycin + cyclophosphamide + 5-FU.

[c] Adriamycin + DTIC; adriamycin + DTIC + cyclophosphamide + vincristine.

Adriamycin has been used on a wide range of dose schedules. The following are the initial adriamycin dose schedules for all phase IV.



TEXT-FIGURE 2.—Investigational approach for an active drug.

National Cancer Institute, Milan, Italy (Dr. Gianni Bonadonna)

1) 0.4–0.6–0.8 mg/kg/day for 4 days; rest for 3 days; then weekly
2) Same doses every other day for 4–6 days; then weekly
3) 0.4–0.6–0.8 mg/kg/day for 3 days; no treatment for 4 days; then treatment for 4 days
4) Same doses as above for 3 days; no treatment for 7 days; treatment repeated
5) 25 mg/m²/day for 3 days every 3 weeks

TABLE 1.—*Antitumor activity spectrum of adriamycin*

Established activity	Possible activity	Unresponsive	Data inadequate
Breast adenocarcinoma	Stomach adenocarcinoma	Large bowel adenocarcinoma	Pancreatic adenocarcinoma
Soft tissue and bone sarcomas	Ovarian adenocarcinoma	Malignant melanoma	Squamous cell carcinoma of esophagus
Bladder adenocarcinoma	Prostate adenocarcinoma	Renal cancer	Endometrial adenocarcinoma
Bronchogenic carcinoma	Squamous cell carcinoma of cervix		Brain tumors
Testicular carcinoma	Squamous cell carcinoma (head and neck)		Chronic leukemias
Thyroid carcinoma	Hepatoma		
Pediatric solid tumors	Multiple myeloma		
Malignant lymphomas			
Acute leukemias			

Roswell Park Memorial Institute, Buffalo, New York
    1) 17.5–35 mg/m^2/day for 2–3 days; no treatment for 3–4 days; treatment repeated
    2) 20 mg/m^2/day for 4 days
    3) 40 mg/m^2/week
M. D. Anderson Hospital, Houston, Texas
    1) 20–30 mg/m^2/day for 3 days every 3 weeks
    2) 20–35 mg/m^2/week for 9 weeks
    3) 60–105 mg/m^2; single dose every 3 weeks
Sloan-Kettering Institute, New York, New York
    1) Dr. Irwin Krakoff: 0.5 mg/kg/day for 3 days; 4 days' rest; treatment for 3 days; etc., until toxicity or response
    2) Dr. Charlotte Tan: *a*) 0.5 mg/kg/day for 4–6 days; *b*) 0.25–0.5 mg/kg every 6 hours for 24 hours; *c*) 1 mg/kg/day for 2–3 days; *d*) 2 mg/kg (single dose)
Institut Jules Bordet, Brussels, Belgium (Dr. Yvon Kenis)
    1) 75 mg/m^2 single dose every 3 weeks

However, efforts to improve the TI have not been highly successful. From the standpoint of efficacy, it is impossible with the available data to state that one schedule is superior. There is a nearly total lack of controlled comparisons of schedules, but the mass of uncontrolled data appears to indicate rough comparability of efficacy on all the commonly used schedules. Kenis et al. (*28*) were among the first to report that a large single dose every 3 weeks gave diminished stomatitis, a fact also observed by other investigators. Currently, the recommended schedule is 60–75 mg/m^2 administered as a single rapid infusion and repeated every 21 days. This approach is supported by pharmacokinetic studies in man (*22–25*). An alternative dose schedule performed in pediatric therapy is 30 mg/m^2 in a single iv dose on 3 successive days and repeated every 4 weeks.

Adriamycin is active against metastatic adenocarcinoma of the breast. When 9 series from the literature are analyzed (*24, 29–36*), objective responses of greater than 50% reduction in tumor size occurred in 96 of 235 treated patients, for an overall response of 41%. This activity compares favorably with that of other active agents tested alone against breast cancer (*37*). The best chemotherapeutic treatment of advanced breast cancer involves drug combinations; many of these have given response rates higher than 50% (*38*). Adriamycin combinations have been vigorously evaluated, and response rates of 84% were reported by Salmon and Jones (*39*) using a combination of adriamycin and Cytoxan; Blumenschein et al. (*40*), who used the same two drugs plus 5-fluorouracil (5-FU), reported a 72% response rate. At present, no adriamycin combination is clearly superior to the standard combinations such as CMF (Cytoxan, methotrexate, 5-FU) developed at the National Cancer Institute (NCI) or CFP (Cytoxan, 5-FU, prednisone) developed at the Mayo Clinic, Rochester, Minnesota. Current trials involve controlled comparisons of adriamycin combinations against those not containing adriamycin and the tests of sequential administration of two combinations of nonmutually cross-resistant drugs. The ultimate use of adriamycin by oncologists in cancer centers remains to be determined, but the value of the drug has been established.

Until the advent of adriamycin, the use of chemotherapy for bone or soft tissue sarcomas, after the failure of primary therapy with surgery or radiotherapy, had not been very successful. Jacobs (*41*), in a review of the literature, noted that treatment with an alkylating agent (usually cyclophosphamide), actinomycin D, and vincristine given alone or in combination yielded an objective response rate of 24% in 238 patients. When childhood rhabdomyosarcomas, which are relatively sensitive to chemotherapeutic agents, are removed from consideration, this response rate was only 18% (32/179). There were few long-term remissions.

Adriamycin has been active against sarcomas in both adults and children (*24, 30, 34, 42–44*). The overall response rate is 25% (69/279) for sarcomas of all types and is roughly equivalent for soft tissue sarcomas (27/103 = 26%) and bone and joint sarcomas (23/76 = 30%).

Gottlieb et al. (*45*) studied adriamycin in combination with 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC, DIC), another experimental drug having slight activity against sarcomas (*46*). The drugs in this combination are synergistic in L1210 and P388 murine tumor model systems (*47*). Clinically, both drugs can be given in combined doses nearly equivalent to those used for each as a single agent. Of 200 sarcoma patients currently evaluable, 85 (43%) achieved an objective response. Although there are some minor differences in response rates by cell type, the number of cases per cell type is not sufficient to demonstrate significant differences.

The addition of vincristine to this combination did not improve the induction rate (42% in 107 patients), but may have increased survival (*48*). Recently, Gottlieb et al. (*48*) added Cytoxan to the regimen (now called CY–VA–DIC), which is given iv in courses every 21 days as follows: Adriamycin, 50 mg/m^2, day 1; Cytoxan, 500 mg/m^2, day 1; DIC, 250 mg/m^2, days 1–5; and vincristine, 1 mg/m^2, days 1–5.

To date, 82 patients are evaluable; there have been 14 complete and 35 partial responses for a 60% overall response rate. The median duration of response will be greater than 6 months, with 43/49 patients (88%) still in remission. The dose-limiting toxicity has been transient leukopenia in one-third of the patients, but serious infections have been rare. This combination regimen appears to represent a significant advance in the therapy of those with metastatic sarcoma.

Currently, a wide range of combinations with adriamycin is being investigated, and only time will tell which combination, if any, will be superior to the two-drug combination of adriamycin plus DTIC.

The chemotherapy of advanced osteosarcoma has improved considerably with the administration of adriamycin alone or in combination with other agents. Cortes et al. (*44*) at the Roswell Park Memorial Institute demonstrated a significant response rate of 41% (7/17) for adriamycin alone in metastatic osteosarcoma. Rosen et al. (*49*) reported objective regression in 54% (7/13) of patients with metastatic osteosarcomas treated with a combination of adriamycin and high doses of methotrexate followed by rescue with citrovorum factor.

In the hope of increasing cure rates for osteosarcoma, adriamycin is being studied as a surgical adjuvant. Mortality in osteosarcoma results from metastases in 80–95% of patients with X-ray evidence of pulmonary metastases occurring in a median of 9 months from initial diagnosis (*50*).

The Acute Leukemia Group B (Scarsdale, N.Y.) has given adriamycin to 21 patients with osteosarcoma within 2 weeks after amputation of the primary lesion. Of the 21 patients, 19 were alive 1+ to 32 months (median, 9+ mo) at the time of the last full report (*51*). Five of the 21 had relapsed, 2 with local and 3 with pulmonary metastases. By life-stable analysis, 71% would be expected to be free of pulmonary metastases in contrast to 30% in a large historic control series. The data demonstrate an effect of adriamycin in delaying gross metastases from osteosarcoma, but large-scale follow-ups are required before a definitive statement on survival is made.

At M. D. Anderson Hospital (*52*) a 4-drug adjuvant chemotherapy called CONPADRI-I [cyclophosphamide, vincristine (Oncovin), L-phenylalanine mustard, and adriamycin] has been used as the primary treatment following surgery in 18 children with osteosarcoma. Ten (55%) of these children have been free of disease 15 months or longer from the time of amputation; this compares favorably to the historic control, 29/33 (88%) of whom developed metastases within 15 months from diagnosis. The regimen was recently modified to include three pulses of massive doses of methotrexate with citrovorum factor rescue (COMPADRI-II = CONPADRI-I plus methotrexate with citrovorum factor rescue).

At Childrens Hospital, Stanford University, Palo Alto, California, Wilbur et al. (*53*) devised a treatment consisting of the following drugs given 3 weeks apart and cycled every 9 weeks: 1) 50–300 mg methotrexate/kg infusion followed by citrovorum factor rescue; 2) 60 mg adriamycin/m^2 given iv plus 2 mg vincristine/m^2 given iv; and 3) 15 mg cyclophosphamide/kg/day for 7 days, given iv or by mouth, plus 2 mg vincristine/m^2 given on days 1 and 7. Of 5 patients who received this regimen after amputation, 4 were free of disease at 12+, 12+, 10+, and 7+ months.

At this time, the potential of adriamycin alone or in combination with other drugs to increase survival when used as an adjuvant to surgery in osteosarcoma appears quite high.

Hodgkin's disease is another tumor that is highly responsive to adriamycin. This is a drug-sensitive tumor, and a wide range of antitumor agents can induce a high response rate (*54*). Combination chemotherapy is now the therapy chosen for the advanced stages of the disease, with the "MOPP" [nitrogen mustard (Mustargen), vincristine (Oncovin), prednisone, procarbazine] regimen developed by DeVita et al. (*55*) being the standard combination. Seventy patients with refractory Hodgkin's disease have been treated with adriamycin for a 30% response rate (21/70) (*24, 30, 34, 35, 43, 56*). Currently, the Southwest Oncology Group is attempting to incorporate adriamycin into the primary combination approach, whereas Bonadonna et al. (*57*) devised a new 4-drug combination called ABVD (adriamycin, bleomycin, vinblastine, and DTIC) that is not mutually cross-resistant to MOPP and possibly has equal activity.

Adriamycin used alone against refractory lymphosarcoma has induced an objective response rate of 37% (20/54) (*24, 30, 34, 35, 43, 56, 58*), which compares favorably with the activity of other single agents (*59*). In cases of histocytic lymphoma (reticulum cell sarcoma), adriamycin alone has induced an objective response rate of 65% (37/57) (*24, 30, 34, 43, 56, 58, 60*). Most of the adriamycin-treated patients had advanced disease and probably had failed to respond to combination chemotherapy, indicating a lack of cross resistance for adriamycin with the standard active agents. A wide variety of drug combinations involving adriamycin have been tested or are now being tested. Among the most successful are those of McKelvey et al. (*61*), who reported an 85% response rate with a combination of adriamycin, cyclophosphamide, vincristine, and prednisone, and 83% with adriamycin, vincristine, and prednisone. The optimal combination remains to be developed.

In urinary bladder carcinoma, chemotherapy has been neglected. Adriamycin has been evaluated in 62 patients, with an overall response rate of 35% (21/62) (*30, 34, 36, 43, 62*); this significant activity is being further examined in several studies.

In thyroid cancer, adriamycin has been studied more extensively than any other antineoplastic agent. Thus far, there is a 30% response rate (14/46) in patients with advanced refractory, metastatic thyroid carcinoma, but analysis in different cell types is not available (*29, 34, 35, 63, 64*).

Forty-six patients with neuroblastoma have been treated with adriamycin and 15 (33%) have shown objective responses (*29, 34, 35, 43, 58, 62, 65*). Although this activity is less than that reported (*66–68*) for cyclophosphamide and vincristine (singly or in combination), the cases treated with adriamycin were unresponsive to prior therapy. Using a combination of adriamycin, cyclophosphamide, vincristine, and methotrexate, Bonadonna et al. (*34*) reported 4 responses in 9 patients; other combination studies are in progress.

Wilms' tumor is another pediatric solid neoplasm in which activity has been observed (7/17 = 41%) (*30, 34, 42, 43, 58*).

In 173 refractory cases of acute lymphocytic leukemia, adriamycin has been administered; 44 (25%) had complete remission (*24, 34, 35, 43, 58, 69–71*). Acute lymphocytic leukemia in children is very responsive to chemotherapy; daunorubicin (the earlier analogue of adriamycin) is the standard anthracycline antibiotic in most combination regimens used in the United States.

Daunorubicin alone produces complete remissions in nearly 50% of previously untreated patients with acute myelocytic leukemia (*72*). Combination chemotherapy in regimens such as cytosine arabinoside and thioguanine (*73*) or cytosine arabinoside and daunorubicin (*74*), which induce a complete remission rate greater than 50%, is the current accepted therapy. With adriamycin, the complete remission rate is 26% (27/102) (*24, 34, 42, 43, 62, 69–71, 75, 76*). Most of these patients had advanced disease and had received prior chemotherapy. McCredie et al. (*77*) (M. D. Anderson Hospital and Tumor Institute) induced a complete remission rate of 85% (23/27) in adult leukemia with a combination of adriamycin, vincristine, prednisone, and cytosine arabinoside (Ad-OAP). Further studies are in progress, but daunorubicin is still used in most cooperative studies.

Although adriamycin can shrink masses of bronchogenic carcinoma, it shares the problem of all chemotherapeutic agents in this disease—symptomatic improvement and objective tumor regression have a minimal influence on survival (*78*). When objective tumor regression greater than 50% was evaluated from 11 series in the literature, the overall response rate was 19% (49/264), with a range of 0–50% (*24, 28, 30, 35, 42, 43, 79–81*). The effectiveness of adriamycin in relation to other single agents can be evaluated by objective response, but the data on adriamycin are insufficient to judge survival as a response variable. Within the limitation of historic comparisons in which data on performance status and prior chemotherapy and radiotherapy are not always reported and results include regressions less than 50%, adriamycin ranks among the most effective single drugs in the data for over 5,000 cases reported by Selawry and Hansen (*78*).

Adriamycin is being evaluated in a variety of combinations to increase the response rates in bronchogenic carcinoma. The Veterans Administration Lung Cancer Study Group is evaluating adriamycin plus cyclophosphamide, whereas the Central Oncology Group is piloting a study of adriamycin plus 1,3-cis(2-chloroethyl)-1-nitrosourea (CCNU) and hexamethylmelamine. AT M. D. Anderson Hospital, an intensive five-drug combination [bleomycin, adriamycin, CCNU, Oncovin, and nitrogen mustard (BACON)] has been developed by Livingston et al. (*82*). This regimen was given to 31 patients with advanced cancer, including 11 with lung cancer. Tumor regressions greater than 50% have been observed in 8 of these cases, including one complete response.

At this time, no combination has been established as clearly superior to single agents.

TOXICITY

The toxic effects of adriamycin are dose related, predictable, and reversible. The major toxicities are dose-limiting myelosuppression in approximately 60–80% of patients, stomatitis in as many as 80%, nausea and/or vomiting in 20–55%, and alopecia in virtually all cases.

Leukopenia is the predominant hematologic toxicity, and the severity depends on the adriamycin dose and the regenerative capacity of the bone marrow. Thrombocytopenia and anemia occur in the same time frame as leukopenia, but they are not as great a problem. Supportive care for hematologic problems should be available for patients being treated with adriamycin.

Drug-induced stomatitis typically begins as a burning sensation with erythema of the oral mucosa and in 2–3 days may produce frank ulceration, particularly in the sublingual and lateral tongue margins. Retrospective comparison of the incidence of stomatitis as a function of dose schedule suggests that it may be less frequent as the interval increases between doses.

Alopecia involving the scalp, axillary, and pubic hair occurs in almost all patients. Growth of hair usually resumes on cessation of the drug.

Gastrointestinal toxicities evidenced by nausea and occasional vomiting are associated with the drug but rarely limit clinical use. Extravasation during iv administration can produce local tissue necrosis, but normal precautions can prevent this toxicity.

Cardiac toxicity is the unique harmful effect of adriamycin that causes the greatest problem in long-term administration. This toxicity may involve transient electrocardiogram (ECG) abnormalities, definitive cardiomyopathy, or both.

ECG changes associated with adriamycin therapy are reported in 2–30% of the treated patients. These transient abnormalities include supraventricular tachycardias, atrial and ventricular extrasystoles, and ST–T wave changes. The changes usually are transient and occur most frequently in the first few days after drug infusion. Reduction in the R-wave voltage may occur and is usually irreversible; in the series by Cortes et al. (*83*), it was seen in all patients before adriamycin-induced congestive heart failure developed. The transient ECG changes apparently are not of clinical significance, since no significant morbidity or mortality has been reported.

In contrast to the transient changes, the chronic toxicity of drug-induced cardiomyopathy produces both morbidity and mortality to a significant degree. This "pump" failure is dose dependent, but shows no apparent relationship to preexisting heart disease. The clinical presentation and pathophysiology of cardiac damage by adriamycin are indistinguishable from other known cardiomyopathies. Although the speed of the clinical course varies, it is usually a rapidly progressing syndrome of congestive heart failure and cardiorespiratory decompensation including dilation of the heart, pleural effusion, and venous congestion. Reversibility of the heart failure does not appear to be a function of the therapeutic intervention. In fact, Gilladoga et al. (*84*) reported that adriamycin cardiomyopathy may be reversed by conventional medical management.

The pathologic findings are limited to changes visible by electron microscopy. The most dramatic change is a marked decrease in the myocardial fibrils accompanied by mitochondrial changes characterized by swelling, focal membrane thickening, and dense inclusions. Other observations include nuclear degeneration, disorganization of the sarcoplasmic reticulum, and depletion of glycogen granules (*85*). These changes are nonspecific and have been described in other types of cardiomyopathy (*86*).

The overall incidence of congestive heart failure caused by drug-induced cardiomyopathy is 1%, although this is deceptive since the toxicity is related to the total dose administered. If the total dose is kept below 450 mg/m², cardiomyopathy is not observed (table 3). Unfortunately, this limits the amount and duration of drug therapy. The frequency of cardiomyopathy is markedly increased at total doses above 550 mg/m², so that a clinician who exceeds these dose levels must be aware of the high risk and balance it against the risk of discontinuing therapy in rapidly developing malignancy.

TABLE 3.—*Frequency of cardiomyopathy in relation to total adriamycin dose in adults*

Adriamycin total dose (mg/m²)	Number of patients at risk	Number of patients with cardiomyopathy		Frequency (%)	Reference
		Nonfatal	Fatal		
<450	663	0	0	0	(*85*)
<400	75	0	0	0	(*83*)
451–500	23	0	0	0	(*85*)
400–499	11	1	0	9	(*83*)
501–550	31	0	1	3	(*85*)
551–600	14	1	2	21	(*85*)
500–599	4	1	0	25	(*83*)
>600	27	7	6	48	(*85*)
>600	10	5	2	70	(*83*)

Rinehart et al. (*87*), who used preejection period to left ventricular ejection time ratios (PEP/LVET) to estimate left ventricular function, reported that 7 of 8 adult patients receiving adriamycin (300–525 mg/m²) had a significant increase in the PEP/LVET ratio. In contrast, only 1 of 8 patients receiving less than 200 mg/m² developed prolongation of the PEP/LVET ratio. It appears that the systolic time interval may be a sensitive noninvasive indicator in predicting cardiotoxicity, although this requires confirmation.

Jones et al. (*88*) have data supporting echocardiography as useful for the detection of adriamycin-induced heart disease. They performed an ECG analysis (with calculation of the ejection fraction) on 54 cancer patients being treated with adriamycin. Only 1 of 43 (2%) who received less than 400 mg adriamycin/m² had an ejection fraction of less than 0.43, whereas 6 of 13 (46%) who received more than this dose had an ejection fraction of less than 0.43 ($P<0.001$). Only 2 of these patients had clinical findings of congestive heart failure; this indicates a subclinical damage due to adriamycin that can be picked up through echocardiographic analysis.

ADRIAMYCIN ANALOGUES

As soon as the dramatic activity of adriamycin was elucidated, a vigorous search for analogues began by the drug research and development program of the DCT. This has involved a wide range of compounds developed in Western Europe and the Soviet Union. Twelve of these compounds, including 6 that have undergone some degree of clinical trial abroad, have been evaluated by the Laboratory of Experimental Chemotherapy at DCT (table 4).

TABLE 4.—*Daunomycin and adriamycin analogues under evaluation in comparison to parent compounds*

Compound	Source
Cinerubin A (NSC-18334)	Ciba Pharmaceutical Products Inc., Summit, N.J.
Cinerubin B (NSC-18335)	Ciba Pharmaceutical Products Inc., Summit, N.J.
Rutilantin (NSC-100289)	Dr. Ulrich Weiss
β-Rhodomycin (NSC-102813)	E. R. Squibb and Sons, Inc., Princeton, N.J.
Daunomycinone (NSC-109351)	Stanford Research Institute, Palo Alto, Calif.
Adriamycin-14 octanoate (NSC-149584)	Farmitalia Co., Milan, Italy
Rubidazone (NSC-164011) [a]	Rhone-Poulenc, France
Daunomycin–DNA complex (NSC-169533) [a]	Belgium
Adriamycin–DNA complex (NSC-169534) [a]	Belgium
Carminomycin (NSC-180024) [a]	U.S.S.R.
Dubiromycin (NSC-180510) [a]	Rhone-Poulenc, France
Daunomycin-13-formylhydrazone (NSC-180511) [a]	Rhone-Poulenc, France

[a] Have had some degree of clinical trial abroad.

Among the analogues with the most clinical data available is rubidazone, a benzoylhydrazone derivative of daunorubicin. The activity of rubidazone in experimental tumor systems is superior to daunorubicin but not more effective than adriamycin (Goldin A: Personal communication). Rubidazone is less toxic than adriamycin and similar to daunorubicin in cardiac toxicity studies in the hamster. In other in vitro and in vivo animal tests, rubidazone is less toxic than either daunorubicin or adriamycin (*89*). Studies in the rabbit cardiac toxicity model (*90*) indicate that rubidazone does cause cardiac toxicity but at a higher total dose than adriamycin (Young D: Personal communication). This could be deceptive in the prediction of toxicity for man, since much higher doses are used in a single course of rubidazone treatment when compared with adriamycin (*91–93*). A phase I clinical trial in the United States is just ready to begin at M. D. Anderson Hospital.

Both dubiromycin and daunomycin-13-formylhydrazone have received some clinical evaluation in France, but are clinically less impressive than rubidazone (Jacquillat C: Personal communication).

Carminomycin is an analogue of daunorubicin originally isolated from the mycelium of *Actinomadura carminata* by Brazhnikova et al. (*94*) at the Institute of New Antibiotics, Academy of Medical Sciences, Moscow, U.S.S.R. The structure has been described in (*95*). Experimental studies in the United States show that carminomycin has significant antitumor activity but lacks superiority to adriamycin in all systems studied (Goldin A: Personal communication). Clinical trials in progress in the U.S.S.R. show an acute toxicity spectrum not dissimilar to other drugs in this class (Perevodahikova N: Personal communication).

The complexing of adriamycin or daunorubicin with DNA is based on the hypothesis of Trouet et al. (*96*) and DeDuve and Trouet (*97*); they posit that DNA confers lysosomotropic properties on the drugs and enables them to achieve selectively higher incorporation into the neoplastic cells. Experimental tumor studies in Belgium and at the NCI have indicated potentially less toxicity and increased efficacy for the DNA complexes. The experimental picture has been complicated by an NCI formulation of adriamycin–DNA showing less activity than the original formulations of Trouet and his associates, and the NCI is attempting to prepare a new formulation. Clinical studies with both complexes have been undertaken in Europe, but their preliminary nature precludes any judgment at this time. Clearly, additional work should be vigorously pursued on this approach.

The most critical questions in analogue searching after a drug is shown to be active are: *a*) What is the basis for choosing a new analogue for clinical evaluation, and *b*) how should its clinical trial be approached? Three basic approaches are being considered for adriamycin analogues:

I. Experimental tumor data
    A. Superiority over adriamycin
    B. Activity in a system unresponsive to adriamycin
II. Toxicology data
    A. Lack of cardiac toxicity
    B. Lack of marrow toxicity
III. Clinical experience in other countries
    A. Clinical activity
    B. Toxicity

Experimental tumor data are most commonly used in choosing a new analogue in most drug development programs. The same experimental tumor systems used to predict activity of the parent compound can be employed to find superior activity for an analogue. This is most easily accomplished when the parent compound is only weakly or moderately active, as is the case with adriamycin in the leukemia L1210 system. It becomes more difficult when the parent compound is already curative to a high degree in a system such as adriamycin in B16 melanoma and P388 leukemia. A second experimental design would be the screening for activity in systems completely unresponsive to adriamycin. This approach is taken in many analogue drug development programs, which use tumor panels to search for differential tumor spectra (*98*).

A toxicologic model could also be incorporated in the decision-making process for choosing an adriamycin analogue for clinical trial. Since dose-related cardiac toxicity is the major limitation to protracted use of adriamycin, a drug with diminished potential for cardiac toxicity, even with less efficacy, would be highly desirable. Potential models in the rabbit, monkey, hamster, and rat have been postulated to have some degree of predictive value; all the models will be investigated in further detail.

The third major approach in choosing an analogue is examination of the data base of clinical experience in other countries. As stated earlier, six analogues of adriamycin and daunomycin have been clinically evaluated abroad, and the information on activity and toxicity will obviously have a major function in the decision of which ones might undergo widespread trial in the United States.

After an analogue has been selected for clinical trial, the strategy of testing becomes of paramount importance and must be designed from both the standpoint of efficacy and toxicity. The efficacy of the analogue could be compared to adriamycin in either tumors highly or moderately responsive to adriamycin or in unresponsive tumors. The major problem with highly responsive tumors is that drug combinations involving adriamycin have the highest priority within the treatment strategy for these tumors. It would be unrealistic to expect a valid test of an analogue in patients previously treated with adriamycin in a combination setting. It might be equally unrealistic to pursue a single-agent trial with an unproved analogue when superior combinations exist. The same problems arise to a lesser degree in moderately responsive tumors, and it is not unrealistic to contemplate a single-agent trial with an analogue in previously untreated patients with bronchogenic carcinoma. The tumors unresponsive to adriamycin are relatively easy to use for analogue testing; used alone, however, they might not constitute a fair trial of the predictive ability in the decision-making process.

When evaluation of the cardiac toxicity of a new analogue is brought into focus, the problems are manifold. A physician could maintain patients who are responsive to an analogue as long as relapse is prevented and observe for congestive heart failure. This occurred with adriamycin when physicians knew of the toxic potential of daunorubicin. Although "ignorance is bliss" to some extent, a continued approach in this manner might not be defensible. An attempt could be made to use ECG or other noninvasive measurements, but their past value in the prediction of the chronic toxicity has been questionable.

CONCLUSION

Chemotherapy is now a well-established modality. As with other therapeutic measures, chemotherapy can be curative or palliative in varying degrees, depending on the individual tumor. "Cure" means that the life expectancy of treated cancer patients is the same as "normal" life expectancy—specifically, the same as that of a matched cohort in the general population. Cures or at least possible cures from drugs alone have been obtained in such diseases as choriocarcinoma, Burkitt's lymphoma, acute lymphocytic leukemia, testicular cancer, and Hodgkin's disease (*99*). Chemotherapy combined with X-ray and/or surgery promises cures in many patients with childhood solid neoplasms such as Wilms' tumor, embryonal rhabdomyosarcoma, and Ewing's sarcoma. In other tumors, chemotherapy achieves a significant degree of cell kill, which is reflected in a high rate of objective tumor regression and enhanced survival, although cure cannot be shown at this time. These tumors include adenocarcinoma of the breast and ovary, non-Hodgkin's lymphoma, multiple myeloma, the chronic leukemias, and acute granulocytic leukemia. In many of the remaining diseases, chemotherapy can achieve objective regression of disease with palliative benefit to one-fifth to one-third of the patients treated.

A major approach to improving the cure rates in cancer treatment involves the integration of chemotherapy with the local modalities of surgery and radiotherapy applied against solid tumors. Such an approach has been elucidated by the DCT (*27*). The philosophic base of this combined modality approach is the recognition that surgery and radiotherapy are local modalities that kill tumor cells only where the modalities are applied. They fail to cure many patients, even when they remove all the tumor visible to the naked eye or on diagnostic X-ray film. This failure is thought to be due to disseminated, microscopic disease foci present at the time of surgical excision of the primary tumor that often includes the surrounding tissue and part of the regional lymph nodes.

Chemotherapy, used optimally, has the potential for eradicating the metastatic foci of early disease. In the DCT strategy, the drug regimens showing the highest degree of activity in advanced disease will be the prime candidates for the combined modality approach. The degree of cell kill necessary to shrink a bulky, solid tumor mass by greater than 50% (usually the minimum definition of objective regression) is quite large. If this level of cell kill could be directed against the relatively small tumor burden remaining after surgical excision, perhaps the last neoplastic cell can be eradicated.

Experimentally, it is well established that the best chance of eradicating a tumor mass with chemotherapeutic agents is when the tumor cell population is small, a situation occurring immediately after resection of the primary tumor. The ability of chemotherapy to produce a cure is greater when all visible tumor is surgically excised than after subtotal resection (*100–102*). The inverse relation between tumor cell population and chemotherapeutic cure has been clearly expressed quantitatively in mice bearing the L1210 transplanted leukemia (*103*). Similar conclusions have been reached in experimental solid tumor systems (*104*).

The DCT proposed therapeutic strategy for increasing cure rates in solid tumors involves integration of drugs into combined modalities for primary treatment according to the following sequential approach: Test new drugs and combinations in advanced disease; develop an optimum chemotherapy regimen for primary treatment of disseminated disease; and integrate the optimum chemotherapy regimen into a combined modality approach for primary treatment of local and regional disease. In this scheme, new drugs and drug combinations would be tested in advanced disease, and those showing positive results would move into primary treatment of disseminated disease. The optimal regimen evolved in this situation would then be integrated into a combined modality approach for primary treatment of local and regional disease.

Adriamycin is a major addition to the armamentarium available to the oncologist in this strategic approach. In breast cancer (the sarcomas and lymphomas), combined modality trials with the use of adriamycin in early disease are either under way or planned, and the continued study in advanced disease is of a high order. The DCT is giving high priority to studies of the mechanism of action and pharmacology of the anthracycline antibiotics to improve the TI and uncover superior analogues.

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ADDENDUM

Adriamycin continues to be a drug with an extraordinary broad spectrum of activity. Its activity in adult solid tumors has now been expanded to include gastric, ovarian, and endometrial cancers. In addition, there is evidence for some activity in cancers of the uterine cervix, head and neck, and pancreas. The major thrusts for adriamycin usage continue to be combination chemotherapy for advanced disease and combined modality usage for primary treatment.

Although adriamycin has been combined most effectively with cyclophosphamide alone or with 5-FU as a third drug in therapy for breast cancer, its use in combinations has not increased the complete or overall response rate appreciably above that which can be observed with nonanthracycline-containing combinations. This is both disappointing and perplexing. The use of adriamycin does appear to be improving response duration and survival, however. Several groups are investigating adriamycin-containing combinations for adjuvant use in stage II–III breast cancer after surgery. These studies are early in their follow-up, so little can be said at this time. Their ultimate analysis will be hampered by the use of historical controls in some series and by the lack of a surgery only control in postmenopausal women for all series. An additional concern will be the long-term manifestations of a subclinical anthracycline-induced myocardial damage.

In the sarcoma area, adriamycin continues to be the mainstay of palliative therapy for advanced disease and is currently under study in the adjuvant mode. In gastric cancer, combinations of 5-FU, adriamycin, and mitomycin C are showing response rates in the range of 50%. Apparently this tumor is more responsive to drug therapy than the other gastrointestinal adenocarcinomas. In ovarian cancer, the combination of adriamycin plus *cis*-platinum is proving to be synergistic and, for the first time, high response rates are being observed in women failing a primary chemotherapy regimen. Also, in bladder cancer this combination is increasing the potential for response.

The problem of cardiac toxicity still is of utmost importance as it limits the total dose of drug that can be given, which is a significant factor in adjuvant use of the drug.

The Stanford group has studied right ventricular endomyocardial biopsy, right heart catheterization, and systolic time interval determination pulse in patients treated with adriamycin. Their results in 33 adult patients indicate that adriamycin administration is associated with a dose-related increase in the degree of myocardial necrosis. In 29 patients who received total doses equal to or greater than 240 mg/m^2, they found 27 with degenerative changes that could be identified on biopsy. The pre-ejection period to left ventricular ejection time ratio (PEP:LVET) exhibited a threshold phenomenon and did not begin to increase until a total dose of 400 mg/m^2 had been reached. Seven patients, whose heart failure was due to catheterization, had a significantly greater amount of myocardial necrosis on biopsy than dose-matched controls ($P < 0.01$). Previous mediastinal radiation appears to potentiate the adriamycin-associated degenerative process. Mediastinal radiation, age over 70 years, and concurrent cyclophosphamide administration are the three apparent risk factors for adriamycin-associated heart failure. It is the belief of the Stanford group that dose limitation by combined clinical, noninvasive, invasive, and morphologic criteria offers an advantage over empirical dose limitation or dose limitation by PEP:LVET alone.

Human right ventricular endomyocardial tissues are obtained by a transvenous intracardiac catheter technique. A flexible biot is introduced percutaneously through the right internal jugular vein and advanced across the tricuspid value to the apical portion of the right ventricular septum where four cardiac muscle specimens from 0.15 to 0.30 cm in diameter are taken serially.

The biopsy tissue is fixed immediately in 10% buffered formalin and processed in a conventional manner for light microscopy. Tissue for electron microscopy is fixed immediately in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Although severe cardiotoxic changes can be detected by light microscopy, electron microscopy is required to score the severity of cardiac toxicity accurately. Two types of damage caused by adriamycin have been observed. The first is a cell, totally or partially devoid of myofibrillar content even though the nucleus and mitochondria might be intact. The second type of myocyte damage is vacuolar degeneration. A biopsy score with a scale of 0–3 has been developed at Stanford:

0 = no change from normal.
1 = scanty cells showing early myofibrillar drop out and/or swelling of the sarcoplasmic reticulum.
2 = more widespread changes with groups of cells showing definite myofibrillar drop out, cells showing definite cytoplasmic vacuolization, or both.
3 = diffuse myocyte damage with marked cellular changes and frank necrosis.

The guidelines for stopping adriamycin (because of potential cardiac risk) developed at Stanford are as follows: A 3+ biopsy score is an absolute indication to stop the therapy. A second indication would be a PEP:LVET ratio of more than or equal to 0.45 or an increase of greater than or equal to 0.10 over the pretreatment value if combined with a 2+ biopsy change or a catheterization abnormality, consistent with myocardial dysfunction. The PEP:LVET ratio increase alone without any other evidence of myocardial dysfunction such as a biopsy change or catheterization change is not an indication for stopping therapy.

In the past, the clinical evaluation of new adriamycin analogs would have required a determination of the cumulative dose and the number of courses to reach that dose which resulted in cardiomyopathy and comparing it with adriamycin. This would have 1) required large numbers of patients, 2) suffered from the faults inherent in the use of historical controls, and 3) been questionable in ethical terms. The endomyocardial biopsy techniques enable one safely to determine a dose level that causes pathologic changes at a preclinical level. It gives both an objective and quantifiable end point without having to wait for the patient to develop a life-threatening clinical situation. This technique should now be of prime consideration in the clinical trial strategy for any new analogs.

The endomyocardial biopsy results to date indicate that on the schedules used at Stanford University almost all patients who receive a cumulative dose in excess of 200 mg/m^2 have changes in their myocardial cells. Repeat biopsies have indicated no healing of these lesions more than a year after the last dose of adriamycin was administered. These data have significant implications for the use of adriamycin in the adjuvant situation. We do not know what the clinical end product of the myocardial damage will be 5 and 10 years after therapy. Since some patients treated with surgical or radiotherapeutic adjuvant adriamycin will have been cured by the adjuvant therapy, the long-term toxicity will have to be factored into the final cost versus benefit analysis. The same is true for the malignant lymphomas when adriamycin is part of potentially curative combination regimens. The evidence that concomitant cyclophosphamide and/or radiation therapy to the mediastinum may enhance the cardiac toxic potential of adriamycin lends even further emphasis to this problem.

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Large-Bowel Cancer—The Current Status of Treatment

Large-bowel carcinoma (of the colon and rectum) afflicts more patients than any other malignant disease in the United States, excluding skin cancer. In 1974, it was estimated that there would be 100,000 new cases and 48,000 deaths from this tumor in the United States. The death rate of 19 per 100,000 male patients has changed little in the last 30 years and is second only to cancer of the lung among deaths attributable to cancer (*1*).

Survival rates after surgery have reached a plateau. In the last 20 years the survival statistics of the American Cancer Society (*2*), various clinical cooperative groups (*3*), and the National Institutes of Health (*4*) have indicated little change in the 5-year survival of patients operated on for curative resection, particularly when lymph nodes were positive. Clinical and experimental efforts in solid tumor research during the past few years have proved that *a*) the apparent staging of solid tumors is not accurate because most are disseminated at surgery; *b*) the unrecognizable minimal residual tumor remaining after surgery is responsible for recurrence and death; *c*) in addition to surgery, adjuvant therapy (either immunotherapy, radiotherapy, or chemotherapy) is a necessary adjunct in the treatment of solid tumors; and *d*) adjuvant therapy should be used at the earliest possible time after operation when the most effective therapy can be applied to the least residual tumor.

The major pathway for the spread of large-bowel carcinoma, besides direct extension, is via the lymphatics. Curability is closely related to the presence or absence of metastases to lymph nodes. From 30 to 68% of reported large-bowel cancers encountered surgically have already metastasized to the regional lymph nodes (*5*), with the frequency of diagnosis of nodal involvement depending largely on how meticulous the pathologist is in his search.

Venous involvement has been reported in 15–61% of patients (*5*) and there is a strong correlation between this finding and later death with metastases. The liver and lungs are overwhelmingly the most frequent sites of metastasis from large-bowel cancer; the less common sites include the bone, brain, adrenal glands, and kidneys. Spread by implantation to peritoneum and abdominal wounds is also common. Whether from implantation or unresected neoplasm, local recurrence frequently ensues after resection and is observed with much greater frequency in lesions below (23 to 34%) than in those above (4 to 11%) the peritoneal reflection (*5*).

Death due to large-bowel cancer results from the effect of distant metastases in about half the patients and from local recurrence in the other half. Moertel and Reitemeier (*6*) found the mean duration of life from the time of proof of unresectable cancer or metastases to be 9.5 months (median 7.0 mo) with a range of from 4 weeks to over 6 years.

SURGERY

Radical surgical excision is the only universally accepted curative treatment for invasive large-bowel carcinoma. As Moertel (*7*) pointed out, the therapeutic results of surgical treatment are difficult to interpret due to lack of uniformity in the type of denominator used in the literature. Five-year survivorship may be expressed on the basis of all patients diagnosed, all patients subjected to surgery, all patients with resection of primary lesions, or all patients resected "for cure." The definition of resection "for cure" is all too often derived from a purely subjective separation by the surgeon of his favorable and unfavorable cases. Operative deaths may or may not be deducted from the denominator, and deaths from causes "other than cancer" may or may not be deducted from both numerator and denominator. There are also wide variations in the type of patient and stage of disease that may present to different types of medical centers.

In 1932, Dukes (*8*) devised a classification of bowel tumors based on the characteristics of wall penetration and regional node spread. He was able to correlate prognosis with the extent of bowel wall penetration and node involvement. Later, Kirklin et al. (*9*) and Astler and Coller (*10*) modified Dukes' method by microscopically zoning the depth of tumor penetration in the bowel wall. Dukes' classification A tumors are those confined to the bowel wall, and the 5-year survival rate ranges from 61 to 81%. Type-B lesions invade through the muscularis without nodal metastasis and the 5-year survival ranges from 25 to 64%. Type-C lesions involve metastasis to regional nodes and survival is from 6 to 28%.

Surgical resection is the only effective method of treating these tumors, but it has had only limited success. According to the latest reports of the End Results Group (*11*) as published by NCI for 1960–64, the overall 5-year survival was 41% for men and 46% for women. The effectiveness of resection varied with the

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Editor's note: Periodically the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

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Table 1.—*Five-year survival after surgery in carcinoma of the rectum* (*12*)

Time period	Number of patients	Operated on for cure (%)	Local recurrence (%)	Dukes' stage of tumor (%)			5-yr survival by Dukes' stage (%)		
				A	B	C	A	B	C
1931–40	177	52	7	17	20	63	53	22	5
1941–50	297	63	16	25	29	46	67	15	2
1951–60	356	65	13	27	30	43	79	25	6

extent of the disease. The End Results Group cites 5-year survivals of 66% in men and 73% in women when the cancer was localized. When regional nodal metastases were present, the survival in men was 37%, and in women, 41%. Considerable information is given in table 1 derived from a review of 830 patients with carcinoma of the rectum, with 100% follow-up (*12*).

A significant difference in survival is found, depending on the location of the primary tumor in the colon or rectum, i.e., the prognosis of the extraperitoneal rectal lesions being 10–15% poorer than those in the intraperitoneal colon (*11*). The difference is most evident when regional nodal metastases are present. Patients with lesions in the lower rectum, with regional nodal metastases, have a significantly poorer prognosis than those with tumors higher in the rectum.

RADIOTHERAPY

Radiotherapy has not played a major role in the treatment of large-bowel cancer except in the area of rectal cancer. Since local recurrence is a significant problem in rectal carcinoma, preoperative and postoperative radiation therapy has been proposed to diminish the regional spread of disease.

Preoperative Radiotherapy

Higgins and Dwight (*13*) reviewed the rationale for preoperative radiotherapy in *a*) improving the resectability rate by reducing the bulk of tumor, *b*) minimizing regional metastasis, *c*) minimizing distant metastasis, and *d*) reducing local recurrence at the excision site.

The largest experience with preoperative radiation therapy as a surgical adjuvant in rectal cancer has been reported by the Memorial Hospital for Cancer and Allied Diseases, New York (*14*). Between 1939 and 1951, 1,700 patients with rectal cancer were treated preoperatively with the 250-kV machine at dose levels between 1,500 and 2,000 rads via two anterior and two posterior pelvic ports. An additional perineal port was included for tumors within 10 cm of the anal verge. The 5- and 10-year survival figures were comparable in patients with Dukes' A and B lesions who had received radiation therapy, and in patients who had not received radiation therapy. However, among patients with spread of tumor to the regional nodes, 37% of those who received radiotherapy were alive at 5 years and 27% were alive at 10 years; by comparison, in the nonirradiated group only 23 and 10% survived 5 and 10 years, respectively. The decision as to which patients received radiotherapy and which did not was not made by random allocation.

A subsequent study of carcinoma of the rectum (*15*), which randomized 700 patients from the same institution into preoperative radiation therapy plus surgery or surgical resection alone, revealed a reversal of the figures. In the radiotherapy group, 40% survived 5 years versus 47% in the nonirradiated group with nodal spread of cancer.

A prospective randomized and controlled study to determine the clinical effect of low-dose preoperative irradiation on clinically operable patients with adenocarcinoma of the rectum and rectosigmoid was initiated by the Veterans Administration (VA) Surgical Adjuvant Cancer Chemotherapy Study Group in 1964 (*13*). A preliminary analysis with the use of life table procedures to produce survival curves, because only a portion of the patient population had been followed for 5 years, revealed the following results. Of 199 patients who had a resection other than combined abdominoperineal resection, the 5-year survival for the treated group was 40% compared to 45% in the nonirradiated group. However, in 414 patients undergoing combined abdominoperineal resection, the 18-month survival was 74% in the irradiated group and 64% in the control group; the 5-year survival figure was 46% for the treated group and 30% for the controls. Further analysis of the data revealed no particular benefit in patients with positive regional nodes. However, positive lymph nodes were found in only 27% of the specimens resected in patients who received preoperative radiation therapy as compared with 40% in the control group.

Radiation therapy consisted of a tissue dose of 2,000 rads given in 10 increments of 200 rads over a 2-week interval delivered to the midplane of the pelvis through opposing anterior–posterior portals. Either conventional beam therapy or high energy therapy was used, depending on which was available in the individual hospital. When the tumor was within 8 cm of the anal verge, an additional 500 rads was administered in 50-rad increments through a perineal portal during the same period. Surgery was done as soon as feasible after treatment.

At the present time five major studies use high-dose radiotherapy for carcinoma of the rectum and rectosigmoid.

At the University of Oregon Medical School, 5,000 rads by MV irradiation were given to 52 patients with rectosigmoid carcinoma. There was no control group. Patient tolerance was good, though there was some diarrhea in a small percentage of patients. A preliminary analysis indicated that no residual gross tumor was found in 10 of 51 patients who completed irradiation and resection. Positive lymph nodes were found in 10 of the 51 cases. There was no clinical evidence of local recurrence at the primary site in 51 patients. At the time their report was published, Allen and Fletcher (*16*) found 28 of these 51 alive without evidence of disease. The study was not randomized and it is impossible to assess the long-term survival benefits achieved by the addition of preoperative irradiation.

Another study was conducted at the Yale–New Haven Hospital, New Haven, Connecticut, by Kligerman et al. (*17*). Patients with potentially curable adenocarcinoma of the rectum and rectosigmoid were randomized by a "flip of the coin" method. Patients in the treatment group received 4,400–4,600 rads from a 6-MEV linear accelerator for 4.5 weeks. The irradiation field used anterior and posterior portals and included the primary tumor, adjacent tissue, and the lymph node drainage area in the true pelvis as well as that extending along the superior hemorrhoidal vessels to the level of the second lumbar vertebra. The radiotherapy was well tolerated, though a high incidence of diarrhea was reported.

Surgical resection was done 4 weeks after completion of the radiation therapy. No significant increase in the difficulty of performing the surgical resection or in postoperative morbidity and mortality was reported. Of the 13 patients randomized to receive preoperative irradiation, one died postoperatively; none of the 16 patients treated by resection alone died postoperatively.

The number of patients was too small and the follow-up period too short to provide meaningful information regarding long-term survival. However, examination of the resected specimens revealed that 11 of 16 patients randomized to surgical resection alone had tumor involvement of the regional lymph nodes; only 3 of 13 treated by preoperative irradiation had positive nodes.

Three cooperative clinical groups are currently performing major studies in this area. The Central Oncology Group (COG) is evaluating two dose levels of preoperative radiotherapy (4,000–4,600 rads and 2,000–2,300 rads) versus an untreated control. The VA group is studying an intermittent dose of 3,150 rads, whereas the Eastern Cooperative Oncology Group (ECOG) is evaluating 4,000–5,000 rads with and without 5-fluorouracil (5-FU), without an untreated control group.

The Gastrointestinal Tumor Group of the European Organization for Research and Treatment of Cancer is using 3,450 rads with and without an untreated control.

Postoperative Radiotherapy

Proponents of postoperative radiotherapy believe that this approach has the following advantages:

1) The total extent of tumor with regard to the local lesion, lymph nodes, and distant metastases would be known. Therefore, various groups of patients could be spared the risks and expense of radiation therapy. These would include patients with Dukes' A and B lesions and those with undiagnosed liver metastases before exploration.

2) Postoperative radiation would not interfere with the operative approach with respect to removal of all known carcinoma or placement of clips around residual disease for radiation purposes. Decisions regarding anterior resection versus abdominoperineal resection, partial bladder resection, and extent of lymph node dissection are often made at the time of the operative procedure on the basis of the extent of local and metastatic disease. Tumor involvement of the bladder and lymph nodes may regress after preoperative radiation to the degree that a surgeon would not suspect initial involvement and would perform less than an adequate resection.

CHEMOTHERAPY

Single Agents

5-FU, with a 21% response rate in over 2,000 patients, has been the most extensively studied drug (*18*). The development of this compound provided the foundation stone for the study of chemotherapy in gastrointestinal cancer.

All investigators reporting its use found this agent capable of inducing regression of large-bowel carcinoma. A wide range of approaches has been devised for the administration of 5-FU, including 2-, 8-, and 24-hour infusions; administration by weekly injection or by intensive course followed by weekly injection; administration in 5 or 10% dextrose; and oral use in capsules or liquid form. There is considerable evidence that a change in the duration or frequency of administration alters the intensity of the overall pharmacologic effect of 5-FU, but no definitive evidence that this is accompanied by a therapeutic index superior to that of the loading course approach originally devised by Ansfield and Curreri (*19*). Unfortunately, controlled comparisons between different schedules of administration have not been too common. One is currently in progress in the COG that compares loading course iv, weekly iv, oral loading course, and a low-dose schedule. The preliminary analysis favors the iv loading course but the data are too incomplete for full analysis.[2]

The use of iv and oral 5-FU was also compared by the Western Cancer Study Group (*20*). On a weekly schedule, 190 patients with disseminated, measurable colorectal adenocarcinoma were started at 15 mg 5-FU/kg/week by both routes and escalated to 20 mg/kg/week after 4 weeks if no toxicity was observed. The dose was escalated in 30% of the patients receiving it orally but in only 13% of the group treated iv. A reduction of 50% or greater in the size of measurable lesions was reported in 13% (12/93) of the group treated orally versus 24% (23/97) in the group treated iv. The superiority of the iv route was significant ($P = 0.05$). The mean duration of remission was also superior in the iv group (33.2 vs. 23.9 wk). The incidence of leukopenia or thrombocytopenia was 30% of the iv group versus 12% in the orally treated group.

The overall response rate obtained from the literature on 5-FU (*20*) administered by the loading course approach is 19%, but Moertel and Reitemeier (*6*) noted a phenomenal range of 8–85% in individual studies, despite the fact that the same drug was used by the same method in the same tumor. This remarkable variation should be recalled whenever one attempts to judge the validity of claims regarding a new agent or a new means of administration.

A new fluorinated pyrimidine (Ftorafur) was reported by the Soviet Union (*21*) and clinical trials are in progress in the United States. This drug is pyrimidine-deoxyriboside-N_1-(2) furanidyl/5-fluorouracil; in addition to the pyrimidine ring, it contains a furanidyl ring. Blokhina et al. (*21*) reported 17 regressions (>25% tumor shrinkage) in 71 patients with large-bowel carcinoma. If only the regressions of greater than 50% were considered, the response rate would fall to only 7% (5/71). The drug is less toxic than 5-FU, but this may be due to less aggressive use, as is indicated by the low response rate.

The only antitumor antibiotics evaluated in more than 50 patients are mitomycin C and adriamycin. Mitomycin C was originally isolated and developed in Japan from a culture of *Streptomyces caespitosus* but may soon be commercially available in the United States. Its mechanism of action is thought to be similar to that of the alkylating agents. In 1960, Frank and Osterberg (*22*) summarized the Japanese data which included 50 regressions in 154 patients with gastrointestinal carcinoma. Several investigators in the United States reported activity in gastrointestinal cancer (*23–25*) and Moertel and Reitemeier (*6*) and Reitemeier et al. (*26*) have evaluated it alone and in combination with other drugs. When this drug was used as a single agent, there were six objective regressions in 56 patients with large-bowel car-

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[2] Ansfield FJ: Personal communication.

TABLE 2.—*Nitrosourea therapy in large-bowel cancer (Mayo Clinic)*

Drug	Number of patients treated	Objective response	
		Number	Percent
BCNU	64	8	12.5
CCNU	75	7	10
MeCCNU	40	7	17.5
Total	179	22	12.3

TABLE 3.—*Randomized comparison of BCNU and 5-FU in large-bowel cancer (Mayo Clinic)*

Drug	Number of patients treated	Response		Mean duration of response (mo)
		Complete response (%)	Improvement >50% (%)	
BCNU	24	0	3 (12.5)	1.8
5-FU	25	2 (8)	6 (24)	6.4

TABLE 4.—*Randomized comparison of 5-FU and meCCNU in large-bowel cancer (Mayo Clinic)*

Drug	Number of patients treated	Improvement >50% (%)
5-FU	21	3 (14)
MeCCNU	19	5 (26)

cinoma and a short duration of remission (mean = 3 mo). Although the drug is capable of inducing regressions in large-bowel cancer, its marked potential for marrow toxicity and the hazard of infiltration ulcers limit its use.

Among the random synthetic and miscellaneous agents, only the nitrosoureas are worthy of attention. For many years, four of these agents have attracted interest as drugs for treatment of gastrointestinal cancer. The first was 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (*27*). Subsequently, two BCNU analogues were put into clinical trial: 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and methyl CCNU (MeCCNU). In addition, streptozotocin, an antibiotic with a nitrosourea structure, has been in clinical trial for several years.

Over the past few years, Moertel (*28*) and the Mayo Clinic (Rochester, Minn.) group treated 179 patients with large-bowel carcinoma with either BCNU, CCNU, or MeCCNU (table 2). The responses with BCNU have been short lived, being maintained for a median duration of 2 months and a mean duration of 4 months. The response rate with BCNU as the first treatment was superior to that obtained in patients previously treated with cytotoxic drugs. In a prospective randomized comparison of BCNU and 5-FU, BCNU was distinctly inferior in response rate and duration (table 3).

The Mayo Clinic experience with MeCCNU, the newest of the nitrosoureas, has been favorable to date. In a randomized comparison with 5-FU, MeCCNU seems to be at least equal to, or perhaps slightly better than, the standard fluorinated pyrimidine (table 4).

Combination Chemotherapy

In the past few years various drug combinations were tested in the search for more effective chemotherapy of gastrointestinal cancer. One combination (MIFUCA) (mitomycin C, cytosine arabinoside, and 5-FU) was injected iv every 3 to 14 days. However, results on colon carcinoma at Memorial-Sloan Kettering Hospital, New York, New York, showed no significant improvement over those achieved with 5-FU alone (*29*). Results with 5-FU and Cytoxan, as well as with CYFUCA (Cytoxan, 5-FU, and cytosine arabinoside) were not significantly better than with 5-FU alone. Until recently, no clear benefit of combination therapy was demonstrated in patients with metastatic large-bowel cancer.

Work at the Mayo Clinic on large-bowel cancer has indicated that combinations of 5-FU + BCNU, 5-FU + mitomycin C, or mitomycin C + BCNU do not offer any advantage in therapeutic response over 5-FU used alone (*26*). A recently completed study, however, indicated that the combination 5-FU + MeCCNU + vincristine may produce objective responses in advanced colorectal cancer significantly superior to those obtained with 5-FU (*30*). In a controlled study, a 43.5% objective response rate was obtained with this combination compared to only 19% with 5-FU alone ($P<0.05$).

The superiority of the combination of MeCCNU + 5-FU over 5-FU alone has been shown by the Southwest Oncology Group in a randomized study of 271 patients (*31*). The 5-FU (400 $mg/m^2/wk$ iv) was given either alone or combined with MeCCNU (175 mg/m^2 orally every 6 wk). The response rate to 5-FU alone in large-bowel cancer was 14% (5/36), whereas 38 of 128 patients (30%) responded to the combination regimen. The combination was more toxic to the bone marrow with a median white blood cell nadir of 3.3×10^3 and a platelet nadir of 75×10^3 compared to 5.1×10^3 and 155×10^3, respectively, for 5-FU alone.

Einhorn et al. (*32*) treated 24 patients with large-bowel cancer with the combination of 5-FU + MeCCNU, using 5-FU on a schedule of 800 mg/m^2 as a continuous 24-hour iv infusion in 1,000 ml of 5% dextrose in water for 5 consecutive days. MeCCNU was given orally at a dose of 150 mg/m^2 on the first day of the 5-FU infusion. Three courses were given at 4-week intervals followed by maintenance with oral weekly 5-FU (500–1,000 mg) and MeCCNU (125 mg/m^2 orally) every 4 weeks. Partial remissions occurred in 29% (7/24) of the patients, with a median duration of 6 months (range, 3–9+ mo). There was minimal toxicity consisting of mild nausea, vomiting, and myelosuppression.

IMMUNOTHERAPY

Experimental evidence suggests the existence of immunologic factors in colon carcinoma. Gold and Freedman (*33*) showed that colon carcinoma contains an antigen also present in fetal tissues, i.e., the carcinoembryonic antigen (CEA). In recent years this antigen has occurred in other benign and malignant conditions. It is present in carcinoma of the colon and following the course of disease (*34*), is of some value in the detection of the occurrence of residual tumor. However, fetal antigens have not caused delayed cutaneous hypersensitivity reactions or stimulated lymphoblastogenesis in patients with carcinoma of the colon (*35*).

Animal experiments demonstrated other antigens associated with colon carcinoma that do not cross-react with human CEA (*36*). Likewise, immunochemical studies of human colon carcinoma revealed three different tumor antigens (*37, 38*). Studies by Hellström et al. (*39*), using the colon inhibition technique, have shown that lymphocytes obtained from patients with colon cancer can inhibit the growth of autologous colon cancer cells. These findings suggest the existence of tumor-specific antigens, common to all cancers of the colon, capable of stimulating a cell-mediated response. Hollinshead et al. (*40*), using an extractable soluble antigen of colon cancer, elicited positive delayed cutaneous hypersensitivity reactions in 17 of 19 patients with colorectal cancer. Other investigators showed impairment of cell-mediated immune responses in colon cancer patients by the inhibition of mixed mononuclear cell migration (*41*), and by phytohemagglutinin (PHA) stimulation of peripheral lymphocytes (*42*), or cells of regional lymph nodes (*43*).

An immunotherapy regimen for large-bowel carcinoma should be a proved safe treatment within the capability of the general medical community. Ideally, to increase the scientific merits of the project, the study should be executed in a setting that will permit a critical comparison to chemotherapy and the combination of chemotherapy and immunotherapy. The methanol extraction residue (MER) of BCG (MER–BCG), developed by Dr. David W. Weiss of Hebrew University–Hadassah Medical School, Jerusalem, Israel, is one material that meets these criteria. It is a uniformly active and available clinical preparation undergoing simultaneous testing and comparison to both BCG and *Corynebacterium parvum* in various neoplasms (*44–46*).

MER–BCG is a powerful nonspecific immunostimulant that has been used as an antitumor agent in animal experiments and is an effective immunologic adjuvant for remission maintenance in acute myelogenous leukemia. In work originating in the Mount Sinai School of Medicine, New York, and the Acute Leukemia Group B, MER–BCG is the subject of similar adjuvant studies and controlled comparisons to other potential immunologic adjuvants. Like other nonspecific immunostimulants, it can control only a critical small (10^9) number of cells. In combination with chemotherapy it may produce a synergistic effect.

MER–BCG is an acceptable immunostimulant in terms of its few known minor clinical side effects. The material circumvents the hazards associated with the administration of live BCG, and there is no clinical evidence that MER can potentiate tumor growth or stimulate the production of blocking factors to the disadvantage of patients. Current clinical trials have actively monitored patients to detect any evidence of paradoxical enhancement as a consequence of immunotherapy. Nevertheless, natural examples of tumor enhancement (in vitro) exist and, therefore, studies of immunotherapy must be adequately controlled (*47–50*).[3]

The Mayo Clinic group (*51*) used MER–BCG alone in treating 40 patients with metastatic gastrointestinal carcinoma who had progressed with previous chemotherapy. A total of 2 mg was given intradermally in 5 sites, either once weekly for 8 weeks or every 4 weeks. Local skin reactions ranging from small papules to draining ulcerations were the only significant side effects. These were intolerable to a few patients on the weekly schedule but were well tolerated on the every 4-week schedule.

Despite the advanced nature of their disease, 46% of the patients exhibited increased reactivity to recall skin tests and 82% could be sensitized to dinitrochlorobenzene. Greater than 100% increases in lymphocyte blastogenesis assays were observed with PHA (52% of patients), concanavalin A (60%), and pokeweed mitogen (55%). These effects were more frequent and rapid in onset with the weekly schedule. Of the 33 patients with measurable disease, 3 had greater than 50% regression with associated improvement in symptoms. This is one of the few examples of immunotherapy producing tumor shrinkage in advanced disseminated disease.

COMBINED MODALITIES

Chemoimmunotherapy in advanced disease has been attempted by Engstrom et al. (*52*) at the American Oncologic Hospital, Philadelphia, Pennsylvania. The regimen consisted of 15 mg 5-FU/kg/week iv plus five immunizing doses of BCG (Glaxo strain) intradermally every 2 weeks. Objective response was reported in 6 of 14 patients (43%) and one of these was a complete response. Mild thrombocytopenia ($<100{,}000$) occurred in 6 of 14 patients, and leukopenia with monocytosis was seen in 12. No serious toxic effects resulted from the BCG. Although this high response rate indicates possible synergism, a randomized trial would be required to prove the point. These data indicate that it would be worth considering.

The rationale of chemotherapy as a surgical adjuvant has evolved significantly during the past 15 years. Initially, the use of chemotherapeutic agents was limited to an interoperative or immediate postoperative course to sterilize the clonigenic cells liberated by surgical trauma. This empirical approach was based on early observations of circulating tumor cells and on experimental work in animal model systems showing the beneficial antitumor activity of chemotherapy against these cells. However, little consideration was given to the growth kinetics of primary bowel tumors or metastases, and the drugs used had only a relatively low order of activity in advanced bowel cancer.

Current studies, largely involving 5-FU, use chemotherapy for a longer time in the hope of eradicating the microscopic foci of metastatic disease remaining after surgery. The VA Surgical Adjuvant Cancer Chemotherapy Study Group has recently ceased patient entry on a study (protocol 23) of the effect of prolonged intermittent chemotherapy on the survival of male patients after resection for large-bowel cancer. This study used 5-FU in 5-day courses starting 14 days after surgery and repeated at 6-week intervals for 18 months in patients having curative resections with poor prognosis for cure. The criteria included *a*) positive lymph nodes, *b*) serosal penetration or invasion of perirectal fat, *c*) blood vessel invasion, *d*) lymphatic invasion, and *e*) cancer in other organs removed as part of the resection.

Additionally, the drug was administered to patients with a proved palliative resection defined as the removal of the primary lesion, but with microscopic evidence of residual disease (i.e., cancer at a resection margin or biopsy of tissues not removed in the resection). Although analysis of this study is incomplete, it does not appear that 5-FU significantly increased survival in comparison to that in the untreated control group.

In their current study, initiated in July 1973 (protocol 27), the VA group is following the same experimental design, but the chemotherapy is a combination of MeCCNU + 5-FU. The drugs are administered in 5-day courses, beginning as soon after surgery as permissible by the patient's condition (about 10–14 days after operation), and repeated at 7-week intervals for 1 year. On the first day, oral MeCCNU is given in a dose based on

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[3] Holland JF: Personal communication.

120 mg/m^2 and, on the same day and each of the next 4 days, 9 mg 5-FU/kg is given iv.

The COG recently reported a study (COG 7041) attempting to determine the effect of long-term adjuvant chemotherapy with 5-FU on the rate of recurrence, time of recurrence, and survival in selected patients treated surgically for carcinoma of the colon and rectum. As in the VA study, two groups were treated (i.e., a curative resection group with poor prognosis for cure and a palliative resection group). The COG defines these groups similarly to the VA group. The chemotherapy differed in that 5-FU was given in a single intensive course within 1 month of surgical resection and was followed by weekly 5-FU maintenance therapy for 1 year.

The preliminary results have recently been reported for 175 patients who had either a curative or a palliative resection and were followed for 6 months to 3 years (*53*). Drug toxicity was rarely severe and consisted of nausea and vomiting (44%), diarrhea (46%), stomatitis (23%), leukopenia (53%), and thrombocytopenia (32%). In the curative resection group, 77% (49/64) are alive and free of disease after treatment with 5-FU versus 67% (42/63) in the control group. After palliative resection, 39% (9/23) in the treatment group are alive and free of symptoms versus 25% (6/24) in the control group. The median survival after palliative resection is 46 weeks for the treated group versus 61 weeks for the control. None of these differences are significant.

A cooperative study headed by Dr. L. M. Rousselot of St. Vincent's Hospital, New York, includes several hospitals in the New York City area. The study will compare 5-year survivals obtained by the intraluminal use of 5-FU versus 5-FU given iv at the time of surgery and thereafter. This study is based on Rousselot's data showing that 30 mg 5-FU/kg in 50 ml water given intraluminally at surgery plus 10 mg 5-FU/kg given iv for 2 days thereafter gave a 74% 5-year survival in 60 patients with stages I and II disease and 65% 5-year survival for 21 patients with stage III disease.

Another study evaluating adjuvant 5-FU after curative resection was recently conducted by the group at Nassau Hospital, Mineola, New York (*54*). They reported on 88 patients (48 with stage II and 40 with stage III—staging system not defined). Starting 4–6 weeks after surgery, two courses (500 mg/day for 5 days iv in each course) of 5-FU were given at 3-week intervals on an ambulatory basis. Eighty-six patients (49 with stage II and 37 with stage III) operated on between 1960 and 1965 were used as a historical control group. Five-year survival without evidence of disease was 69.4% in the chemotherapy group compared to 45.1% in the controls. For patients with stage III disease, the 5-year no evident disease (NED) rate was 27% for the control and 63.3% for the treated group. Although the differences are statistically significant, the use of a historical control makes one cautious about ascribing biologic significance to the data. The toxicity from the drug was described as minimal and short lasting, but the effects were not detailed.

The Gastrointestinal Tumor Study Group of the Division of Cancer Treatment, NCI, a contract-supported group of major cancer centers performing combined modality studies on the major gastrointestinal adenocarcinomas, has recently initiated a series of studies in large-bowel cancer. In rectal carcinoma (i.e., the distal edge of the primary $\leq$12 cm from the anal verge), patients who have undergone a curative resection of Dukes' B$_2$ (extension to perirectal fat) and C carcinoma with no gross tumor remaining will be entered into a study comparing *a*) postoperative radiation, *b*) postoperative chemotherapy, *c*) postoperative radiotherapy and chemotherapy, and *d*) no additional treatment (table 5). In patients with colon cancer (>12 cm from anal verge), immunotherapy will be substituted for the radiotherapy in an otherwise similar protocol design. The immunotherapy will be MER–BCG based on the data presented earlier.

TABLE 5.—*Gastrointestinal tumor study group protocol for rectal carcinoma* [a]

Stratifications	Treatment [b]
Type of operation Abdominal–perineal resection Anterior resection	Radiotherapy alone (regimen 1) 4,600 rads in 4.5–5.5 wk Chemotherapy alone (regimen 2) Duration, 18 mo
Sex Male Female	(A) 325 mg 5-FU/m^2/day for 5 days iv on day 1 and every 10 wk thereafter 130 mg meCCNU/m^2 orally on day 1 and every 10 wk thereafter
Dukes' classification B$_2$ C$_1$ ($\leq$4 positive nodes) C$_2$ (>4 positive nodes)	(B) 375 mg 5-FU/m^2/day for 5 days iv on day 36 and every 10 wk thereafter
Para-aortic lymph node biopsy Done Not done	Radiotherapy and chemotherapy (regimen 3) Duration 18 mo (A) 4,600 rads in 4.5–5.5 wk (B) 500 mg 5-FU/m^2 iv on days 1, 2, and 3 of (A)
Time from operation to first adjuvant treatment 21–42 days 43–60 days	500 mg 5-FU/m^2/day for 3 days iv within 7 days of completing (A) (C) 5 wk after (B) 300 mg 5-FU/m^2/day for 5 days iv 100 mg meCCNU/m^2 orally, one dose (D) 5 wk after (C), continuous chemotherapy as in regimen 2, but starting with regimen 2 (B), then 5 wk later with 2 (A), then 5 wk later with 2 (B), etc. No additional treatment

[a] Resection for cure of Dukes' B$_2$ and C rectal lesions.

[b] Randomized.

CONCLUSION

At this time one of the major thrusts in the therapy of solid tumors is the combined modality approach. The major ingredient in this approach is the addition of a systemic modality of treatment (chemotherapy or immunotherapy) which potentially can eradicate tumor foci anywhere in the body to increase the cure rate above that obtainable with the local modalities of surgery and radiotherapy (*55*). Early successes in this area involved pediatric malignancies, i.e., Wilms' tumor, embryonal rhabdomyosarcoma, and Ewing's tumor (*56*), and recent preliminary data show that combined modalities are probably effective in breast cancer (*57, 58*) and osteosarcoma (*59, 60*).

Currently there is reason for cautious optimism in the success of a similar approach in large-bowel cancer. The preliminary data of the COG indicate that 5-FU, which shrinks advanced disease in only approximately 20% of cases, may be able to delay recurrence and hopefully correlate with increased survival. The combination of MeCCNU and 5-FU, as shown by Moertel and his group and confirmed by the Southwest Oncology Group, can give superior results in advanced disease when compared to 5-FU alone. It is hoped that this combination, used alone or combined with radiotherapy, will lead to an even more dramatic effect when applied to the microscopic residual disease remaining after surgery. Two trials have recently been initiated to evaluate this postulate and, in addition, the new modality of immunotherapy is being introduced into the picture. The next few years should be a period of high interest to those involved in the therapy of large-bowel cancer.

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ADDENDUM

In 1976, cautious optimism prevailed concerning the potential for therapeutic advances in large bowel cancer. Finally, a combination with activity superior to fluorinated pyrimidines and adjuvant chemotherapy with combination chemotherapy was beginning to be used. Since then, the data have not continued to support an optimistic view. The combination of nitrosourea and 5–FU has not been confirmed as superior to single agents. In recent data from the Mayo Clinic, the combination of MeCCNU and 5-FU gave only a 16% response rate, and when all their studies are analyzed, no survival advantage is demonstrated by the combinations over single agent therapy.

The adjuvant therapy studies for colon cancer are now numerous, with many patients entered into various investigations by major cooperative groups. The major combination being tested is again MeCCNU and 5-FU. At this time, no study has accumulated a follow-up long enough for one to say anything meaningful about the analysis.

Most research in rectal carcinoma now involves postoperative radiation therapy. Earlier studies by several groups with preoperative radiation have been closed for poor case accrual because many surgeons have difficulty in accepting the delay imposed by this approach. None of the postoperative radiation studies have reached the point of meaningful analysis.

Immunotherapy has not been established yet as a beneficial modality in this disease nor have prospectively controlled studies shown the immune modulating approach to be advantageous.

New drugs with activity against this common cancer are desperately needed. Scientists in the NCI drug development program are now using rodent transplantable and heterotransplant colon models in their new screen, which they hope will pick up new drugs with activity in large bowel cancer.

GUEST EDITORIAL

by John S. Macdonald, M.D.
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Current Diagnosis and Management of Pancreatic Carcinoma

The incidence of carcinoma of the pancreas has increased in the United States during the past 3 decades, particularly in black males. It presently ranks as the fourth (*1*) most common cause of death by cancer, exceeded only by lung, large bowel, and breast cancer. Over 19,000 cases are diagnosed each year, and essentially all patients can be expected to die of their disease. In recognition of the importance of the disease as a health problem in this country, a National Pancreatic Cancer Project has been established. Several epidemiologic studies have been done in an attempt to identify specific etiologic factors that may be responsible for the increasing incidence of this disease. There is evidence (*2*) that chemical carcinogens may be important causative agents. Studies (*3*) on some members of the American Chemical Society suggest that exposure to certain chemicals increases the risk of pancreatic cancer. Furthermore, cigarette smoking and a diet high in cholesterol have demonstrated a positive correlation with the occurrence of pancreatic cancer. Animal studies have clearly demonstrated that the pancreas is a target organ for chemical carcinogenesis. *N*-Methyl-*N*-nitrosourea (*4*) and di-*N*-propylnitrosamine (*5*) can produce adenocarcinoma of the pancreas in specific rodent species.

Pancreatic cancer is rarely diagnosed at an early stage. The initial symptoms may be nonspecific and are frequently disregarded until anorexia, weight loss, pain, or jaundice become overtly manifest. An early diagnosis requires a high index of suspicion on the part of the physician and the willingness of the patient to undergo a series of diagnostic procedures for what might otherwise appear to be mild complaints.

CLINICAL MANIFESTATIONS

Pain is an important feature of pancreatic carcinoma (*6–9*). It typically presents in the epigastrium, with radiation to the back in approximately 25% of patients. It increases in severity and frequently requires the use of narcotics and neurosurgical palliative procedures in carefully selected patients.

Anorexia and weight loss commonly accompany pancreatic cancer and may become the most debilitating feature of the disease. Occasionally, the weight loss may be associated with signs of pancreatic exocrine insufficiency and malabsorption. No data as yet exist to allow for an accurate estimate as to how frequently malabsorption is an important clinical problem and to what magnitude therapy further compromises pancreatic function. Pancreatic endocrine insufficiency with resultant diabetes has been found in less than 20% of patients.

Obstructive jaundice can be expected to occur in greater than 75% of individuals in whom the tumor arises in the head of the pancreas and may be associated with a palpable gallbladder (Courvoisier's sign). Jaundice may be "painless," but more frequently is preceded by, or coexists with, abdominal discomfort.

Unfortunately, by the time physical signs are demonstrated, the disease is generally inoperable because of local spread or distant metastasis. Because of the retroperitoneal location of the organ, physical examination is often unrewarding in patients presenting with early disease. Only 20% of individuals with pancreatic cancer will have a palpable epigastric mass. Hepatomegaly may result from biliary tract obstruction or liver metastasis. The presence of an abnormal supraclavicular lymph node may be an initial sign of distant metastasis.

DIAGNOSIS

A great deal of clinical investigative effort has been directed toward the definition of the usefulness of available or newly developing diagnostic procedures for the detection of pancreatic cancer. Routine hematologic and clinical chemistry tests are too insensitive and nonspecific to be of diagnostic value. As a result, radiographic procedures have been the mainstay for diagnosis.

The conventional upper gastrointestinal series (UGI) has low diagnostic accuracy (*10*) and most often only detects a large carcinoma that has caused gross pancreatic enlargement and secondary infiltration of the surrounding structures. Abnormalities commonly found on UGI involve the posterior gastric wall, retrogastric space, antrum, and duodenal loop. Hypotonic duodenography (*10–12*), a modification of the UGI, has a diagnostic accuracy of 75% in cancer of the pancreatic head and about 90% in ampullary carcinoma. Unfortunately, most patients are already symptomatic at the time of this procedure. Hypotonic duodenography is not sensitive enough and cannot be used to evaluate lesions of the body and tail of the pancreas.

Ultrasonography, a new noninvasive procedure, has been useful in detecting pancreatic disease. Typical echo patterns have now been defined for acute pancreatitis, fibrosis, and tumors of the head, body, and tail of the pancreas (*13, 14*).

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

^{75}Se-selenomethionine (*15*) scanning allows the assessment of size, shape, and position of the pancreas and defines the extent of morphologic damage. It can detect neoplasms over 2 cm and is particularly helpful for lesions in the body and tail of the pancreas. An unequivocal normal scan is a reliable finding to exclude pancreatic disease. However, abnormal scans pose difficulties in defining pathologic entities (*16*). Differentiation between pancreatitis and diffuse involvement of the pancreas with carcinoma is, at best, difficult; furthermore, 30% false-positive findings have been reported. Concentration of the isotope in the liver with overlap interference has been a problem that is being resolved with new photosubtractive techniques. Continued investigation into this field to improve the specificity of isotopes and resolution may make this a valuable screening procedure.

The use of computerized axial tomography in the detection of pancreatic masses is currently undergoing evaluation at Georgetown University Hospital. The ACTA-scanner (*17*) has provided good definition of pancreatic masses in several patients with pathologically proven pancreatic cancer. This research tool is currently undergoing a prospective evaluation to determine its usefulness in both the detection and following of patients with unresectable pancreatic cancer as compared to other noninvasive techniques, e.g., ultrasound.

Endoscopic cholangiopancreatography (ERCP), performed with a fiberoptic duodenoscope with cannulation of the ampulla of Vater and retrograde injection of contrast material into the pancreatic ducts, is a new and developing technique (*18*). This procedure has been used most frequently on patients with advanced disease, and as a result its value in the early detection of pancreatic carcinoma is unknown. The overall accuracy of this procedure in the diagnosis of pancreatic carcinoma is still being determined, but some authors (*10, 18*) claim a 90% yield. The major findings of pancreatic cancer by ERCP include pancreatic duct stenosis (solitary or multiple), delayed contrast outflow, ductal occlusion or displacement, necrotic cavity formation, and deformity of the common bile duct by a tumor in the pancreatic head (*19–22*). Combined with ERCP, investigators have added carcinoembryonic antigen (CEA) determinations on pancreatic juice (*23*); cytology of duodenal juice, pancreatic juice, and bile (*24*); and secretin testing with collection of ductule fluids and analysis of bicarbonate levels (*25*). One report claims a 79% (*24*) accuracy with the combination of ERCP and cytology; however, limitations arise in patients with early-stage lesions, neoplasms located in the body and tail of the pancreas, and in those in which exocrine activity is significantly impaired. Combined ERCP–CEA–secretin testing is a new diagnostic approach undergoing evaluation. Duodenal drainage techniques with the use of the secretin test have been positive (*26*) in 85–95% of patients with pancreatic carcinoma that involves the head of the organ, but have not been helpful at an early stage of disease when cure is possible.

Transhepatic cholangiography may be of value in demonstrating compression, distortion, or obstruction of the common bile duct by tumor in an enlarged head of the pancreas.

Selective and superselective angiography (*27, 28*) are highly accurate procedures that allow differentiation of carcinoma of the pancreas from pancreatitis in approximately 85% of cases, and 1- to 2-cm cancers may be detected. Magnification techniques and the pharmacoangiographic (*27, 29*) use of vasoconstrictors and vasodilators significantly improve diagnostic confidence and accuracy; such techniques allow differentiation of the normal and abnormal pancreas in 95% of individuals and a diagnostic accuracy up to 95% in pancreatic adenocarcinoma (*27, 30*). Many angiographic features of carcinoma have been described (*10, 27, 31*). These include arterial narrowing and encasement, which suggests compression or invasion of the vessels by the neoplasm (*29, 30*); neovascularity, an uncommon finding consisting of small, tortuous vessels in fine network; and arterial obstructions of the celiac, superior mesenteric, hepatic, splenic, gastroduodenal, or small intrapancreatic branches. Tumor opacification (i.e., tumor blush) is rare. The splenic, portal, and superior mesenteric veins are often involved, especially in tumors of the body and tail of the pancreas, which may grow to large sizes before diagnosis because of a paucity of early symptoms. Vascular displacement is uncommon, since the carcinoma infiltrates rather than displaces surrounding tissue (*31*). Hepatic metastases and gallbladder enlargement can be evaluated by configuration of the hepatic and cystic arteries. In addition to its diagnostic value, pancreatic angiography is useful for predicting tumor resectability (*27, 32, 33*). Patients with encasement limited to intrapancreatic arteries and tumor vessels and involvement of the gastroduodenal artery but not the extrapancreatic arteries are amenable to surgical exploration and possible resection.

The possibility of the use of circulating marker substances for diagnosis and follow-up of patients with pancreatic carcinoma is being examined. Ona et al. (*34*) demonstrated elevated CEA levels (>2.5 ng/ml) in the majority of a series of patients with pancreatic cancer. The elevation of CEA was correlated with extent of disease in 71% of patients with metastatic disease who had CEA levels greater than 10 ng/ml, whereas all patients without apparent metastasis had CEA levels less than 9 ng/ml. Unfortunately, CEA lacks specificity and may be elevated in patients with nonmalignant disease of the pancreas. Delwiche et al. (*35*) showed that 18 of 42 (43%) patients with pancreatitis had elevated CEA levels. The usefulness of monitoring CEA to follow the therapy of pancreatic cancer has not been clearly defined.

α-Fetoglobulin is a fetal protein that may be elevated in the sera of patients with hepatocellular carcinoma and embryonal cell carcinomas. Recently, McIntire et al. (*36*) demonstrated elevated α-fetoglobulin (>40 ng/ml) levels in 25% of patients with pancreatic carcinoma. This relationship between α-fetoglobulin and pancreatic carcinoma deserves further investigation to establish the efficacy of this serum marker in following the course of patients with cancer of the pancreas.

Both CEA and α-fetoglobulin are nonspecific markers; the ideal tumor marker would be a specific tumor antigen circulating in patients with pancreatic malignant disease. In 1974, Banwo et al. (*37*) demonstrated evidence for an oncofetal antigen circulating in the sera of patients with pancreatic cancer. Homogenates of fetal pancreas were used to immunize rabbits, and the resultant antisera were absorbed with human albumin and adult pancreas. With Ouchterlony gel diffusion techniques, the absorbed antisera formed precipitin lines with the sera of 36 of 37 patients with pancreatic carcinoma. No precipitin lines were seen in 38 controls including patients with pancreatitis, obstructive jaundice, cirrhosis, carcinoma of the colon, gastric carcinoma, and hepatoma. This work (*37*) needs to be confirmed with more sensitive techniques including radioimmunoassay. However, a sensitive and specific test for circulating specific oncofetal antigen or tumor-specific antigen offers the hope for increased detection of individuals in whom resection with curative intent is possible.

Although these indirect means for detection of pancreatic cancer are becoming more useful, definitive diagnosis requires tissue confirmation of malignancy. In the past, surgical dogma always held that biopsy of the pancreas was a hazardous procedure with an unacceptable rate of morbidity and mortality (*38, 39*). Complications cited in the literature include pancreatic cutaneous fistulae, pancreatitis, and postoperative wound infection in as many as 25% of patients. Surgeons have been tempted to make the "clinical diagnosis" of pancreatic carcinoma solely from inspection and palpation of the organ at the time of laparotomy. However, the forms of postoperative therapy currently used mandate for a definitive diagnosis and exclusion of chronic pancreatitis as the etiology of a pancreatic mass. Recently, a large series (40) of patients at the Mayo Clinic were reported to have experienced little morbidity after pancreatic biopsy. Of 527 patients in the total series, 112 underwent exploratory laparotomy with pancreatic biopsy as the only surgical procedure. Of these 112 patients, 64 had Vim Silverman needle biopsies and 48 had wedge biopsies. Seven complications occurred including postoperative pancreatitis (2 patients), subphrenic abscess (1 patient), and wound infection (4 patients), for a total complication rate of 6.2% with no deaths. This compared favorably to a complication rate of 4.1% secondary to pancreatic biopsy in 415 patients who had biopsies followed by definitive surgical procedures. The frozen-section interpretation of pancreatic biopsy specimens was highly satisfactory, with only one false negative in 201 patients interpreted as having benign specimens on frozen section. Thus tissue diagnosis by pancreatic biopsy can clearly be accomplished at laparotomy with very acceptable morbidity. The morbidity (*40*) of biopsy may be further reduced in lesions of the head of the pancreas if transduodenal needle biopsy is the only biopsy procedure performed.

Less invasive means of pathologic diagnosis of pancreatic carcinoma are undergoing initial trials with promising results. Twenty cases of pancreatic carcinoma (*41*) diagnosed at peritoneoscopy have recently been reported from investigators in Germany. Both Menghini needle biopsies and thin-needle aspirates for cytology were done under direct peritoneoscopic visualization with no evidence of bleeding, fistula formation, or elevation of serum amylase.

Thin-needle cytologic aspiration of pancreatic tissue has also been done under ultrasonic guidance (*42, 43*). Smith et al. (*42*) aspirated pancreatic tissue for cytology from 6 patients who had pancreatic tumors. Five of the 6 had positive cytology for carcinoma, and the procedure resulted in no morbidity. It is encouraging that rapid progress is being made in relatively noninvasive techniques capable of making a definitive pathologic diagnosis of pancreatic carcinoma because these techniques may in the future aid greatly in the development of intelligent procedures for patient diagnoses and management.

TREATMENT

After pancreatic carcinoma has been diagnosed, a plan of therapy appropriate to the stage of disease must be developed. It cannot be stressed too heavily that a combination of treatment modalities is the most effective manner to approach the management of most patients with pancreatic cancer.

Surgery

Surgery must be considered the only modality presently available with the potential for curing carcinoma of the pancreas. For all practical purposes, the only lesions that lend themselves to definitive management by surgery are small tumors of the head of the pancreas which result in early obstructive jaundice. Two curative operations may be performed: 1) Radical total pancreatectomy which entails duodenectomy, choledochojejunostomy, and retrocolic gastrojejunostomy; or 2) the "Whipple" procedure in which the tail of the pancreas is retained and a pancreaticojejunostomy is performed. Both carry operative mortalities of 10–40% in most reported series (*8, 44–47*). Also, 50% of surgical patients may be expected to have a major postoperative complication (*9*). Although there is some suggestion (*46, 47*) that morbidity may be decreased if total pancreatectomy is performed, thus avoiding the postoperative problems associated with pancreaticojejunostomy, it is doubtful that the significant morbidity and mortality associated with these operations will ever be greatly altered.

Long-term survival statistics after radical pancreatectomy have been discouraging. Most series report 5-year survivals of 10–20% (*44, 48*) for patients undergoing "curative" surgery. This survival rate is less than the operative mortality of many series and emphasizes the importance of careful patient selection for radical pancreatectomy. The procedure should not be undertaken unless the patient has a small localized lesion, is without systemic symptoms, and has been carefully evaluated to rule out metastatic disease.

Although considerably less than 10% of patients presenting with pancreatic carcinoma can be cured, many patients can benefit from palliative surgery and information obtained from exploration. Gastroenterostomy and biliary diversion can remove symptoms of bowel obstruction and jaundice and can result in an increased survival (*7, 45*). The series of Crile (*45*) at the Cleveland Clinic is typical. His conclusions were that radical pancreatectomy does not benefit patients with symptomatic carcinoma of the head of the pancreas and that patients who underwent bypass procedures without resection have a longer survival than those given radical operations. In addition to bypass procedures, careful intraoperative staging with examination and biopsy of suspicious lesions in the liver, mesentery, and peripancreatic fat will supply crucial information in planning postoperative therapy for the patient. The placing of surgical clips around the areas of bulk disease at the time of laparotomy is helpful to the radiotherapist in planning treatment portals.

It is clear that surgery alone has little to offer in the management of pancreatic cancer and that 80–90% of patients (*44, 46*) who are operated on with a high-risk "curative" procedure die within 5 years of recurrent disease. These patients are candidates for an effective adjuvant therapy. Moertel et al. have shown (*49*) that combined 5-fluorouracil (5-FU) and irradiation can produce objective responses in patients with locally unresectable pancreatic cancer, and currently the Gastrointestinal Tumor Study Group (GITSG) (*50*) is conducting a controlled randomized trial utilizing 5-FU and megavoltage irradiation versus no further therapy in patients undergoing partial or complete pancreatectomy with curative intent. Because of the limited number of patients available for such a study, an answer will not be available for several years. Until the results of the GITSG study are completed, we recommend that adjuvant therapy with combined radiation and chemotherapy be undertaken in the surgical patient because of the high rate of relapse.

Radiation Therapy

Many symptoms of pancreatic carcinoma are caused by the local invasion of regional lymph nodes, splanchnic nerves, and vessels, and potentially may be palliated by local radiation therapy. However, several investigators have shown that the disease is relatively unresponsive to conventional (3,000–4,000 rads) megavoltage radiotherapy. Childs (*51*) demonstrated that conventional radiation of 3,500–4,000 rads does not result in increased survival. In addition, little published evidence exists for a significant palliative effect in patients with pain and/or obstructive symptoms. Although 5-FU (*7*) as a single agent has a response rate of approximately 20% and little significant effect on the course of patients with unresectable pancreatic carcinoma, the combination of 5-FU and conventional megavoltage radiation appears to have significant activity. Moertel et al. (*49*) reported a randomized prospective trial in which radiation therapy and radiation therapy plus 5-FU in patients with unresectable pancreatic carcinoma were compared. Each group received radiation therapy for a total dose of 3,500–4,000 rads at 900–1,200 rads/week combined with either iv placebo or iv 5-FU (15 mg/kg) given on each of the first 3 days of radiation therapy. Of 64 patients entered onto this protocol, the mean survival for the patients treated with 5-FU was 10.4 months versus 6.3 months for the patients given placebos. This difference was significant ($P<0.05$) and demonstrates evidence of synergism between 5-FU and conventional radiation therapy.

Radical radiation therapy may have a potential function in the treatment of unresectable pancreatic carcinoma. Haslam et al. (*52*) reported a small uncontrolled group of patients treated at Duke University with 6,000 rads to the pancreatic bed. Over 2 weeks, 2,000 rads in 10 fractions were given; this was followed by a 2-week rest period. This cycle was repeated until the full 6,000 rads were administered. This study reported a 24% 2-year survival, which is highly significant when compared retrospectively to the Mayo Clinic's series of untreated patients in which only 2% of patients survived for a comparable period.

The GITSG (*53*) has recently completed a study that helps define the role of high-dose radiation therapy with or without 5-FU in the treatment of locally unresectable pancreatic cancer. Patients were randomized between treatment with 6,000 rads alone, 4,000 rads + 5-FU, and 6,000 rads + 5-FU. Patients treated with either 6,000 rads + 5-FU or 4,000 rads + 5-FU had significantly improved (35 wk and 28 wk, respectively) median survivals when compared with patients treated with 6,000 rads alone (median survival, 17 wk). Hematologic toxicity was most severe in patients treated with 6,000 rads + 5-FU. This study confirms the finding of improved survival in patients treated with 5-FU and irradiation and suggests that the regimen of 4,000 rads + 5-FU is better tolerated and at least as effective as aggressive combined modality therapy with 6,000 rads + 5-FU. It is also clear that 6,000 rads of external irradiation alone is inadequate therapy for unresectable pancreatic cancer.

Other forms of radiation therapy have generated some interest in the treatment of pancreatic cancer. Interstitial implantation of ^{198}Au, ^{222}Ra, ^{192}Ir, and ^{125}I seeds directly into the locally unresectable pancreatic carcinoma at laparotomy has the theoretical advantage of delivering very high doses (8,000–16,000 rads) to the tumor bed. In a series of 19 patients given implantations at Sloan-Kettering Memorial Hospital (*54*), 80% achieved excellent pain palliation and 60% had relief of obstructive jaundice. The therapy was well tolerated, but this technique does carry the potential danger of producing severe radiation damage to the small intestine, particularly the duodenum. When ^{125}I is used as the radiation source, the prospect exists that involved regional lymph nodes may not receive a sufficient radiation dose to establish control. Before any conclusions on the effectiveness of interstitial radiation can be drawn, the techniques will require evaluation in prospective controlled trials in combination with external radiation and chemotherapy.

Fast neutrons offer another form of radiation that appears, in initial studies, to be of some interest. Neutrons do not require oxygen for effectiveness and, therefore, may be capable of treating large tumor masses with anoxic regions; in addition, neutrons have an increased relative biologic effectiveness when compared to conventional photon (X-ray) therapy (*55*). In a clinical trial of fast-neutron therapy, Catterall (*56*) reported objective tumor regression in 17 of 31 patients with advanced adenocarcinoma of the stomach treated with 1,440 rads in 12 treatments over 26 days. This is the equivalent of 4,300 rads of conventional irradiation, and it was generally well tolerated. In the same series, 5 patients (*57*) with unresectable pancreatic carcinomas were treated with similar doses; all patients experienced excellent pain relief, though objective parameters of response were not evaluated. At Georgetown University Hospital, we have developed a pilot study in cooperation with Dr. Charles Rogers (Mid-Atlantic Neutron Therapy Association) to test the efficacy of combined fast-neutron and 5-FU therapy in the treatment of locally unresectable pancreatic carcinoma. If this program is well tolerated and evidence of patient response is seen, a prospective randomized trial versus standardized therapy with 5-FU and megavoltage radiation will be performed.

The results of the GITSG (*53*) evaluation of 4,000 and 6,000 rads of radiation therapy with and without 5-FU in the treatment of patients with locally unresectable pancreatic carcinoma indicate that 4,000 rads of radiation with 5-FU, as described by Moertel (*49*), must be considered standard therapy. Patients should be maintained on 5-FU therapy after the radiation is completed. When effective combination chemotherapy programs are developed, these may be substituted for 5-FU maintenance.

Chemotherapy

The chemotherapy of advanced carcinoma of the pancreas has posed many difficult problems for the medical oncologist. Patients frequently do not present with objective parameters of followable disease, but nevertheless pursue an intractable downward course. The chemotherapy of advanced pancreatic cancer has been disappointing in the past (*7*). 5-FU has been the standard single agent used by most investigators, and response rates from 5% (*58*) to 67% (*59*) have been reported in uncontrolled trials. Objective response rates with 5-FU in most current series average 20% or less, and the wide variation in response may be due to factors in patient selection, lack of objective criteria for response, and the wide varieties of dosages and schedules that have been used in the administration of 5-FU. There is still major debate among advocates of loading courses, weekly courses, and oral courses of 5-FU concerning which method of administration may be most efficacious. However, a recently reported (*60*) prospective randomized study of 5-FU in gastrointestinal cancer strongly suggests that iv loading is the most effective method of administration. The duration of response in patients with pancreatic carcinoma responding to 5-FU therapy has been short (2–4 months), and no evidence exists that treatment with 5-FU improves survival (*61*).

Few cancer chemotherapy agents have been tested in pancreatic carcinoma, and presently information is adequate for only three drugs or classes of agents in the clinical literature. Along with 5-FU, mitomycin C (*62*) and possibly streptozotocin (*63*) appear to have some activity against adenocarcinoma of the pancreas. Mitomycin C, similar to the chloroethyl nitrosoureas, has a delayed and prolonged myelosuppressive effect, with onset approximately 30 days after initiation of therapy. Repeated courses of treatment can result in cumulative bone marrow injury. Many investigators have utilized "loading" dose scheduling, which produces very significant myelosuppression. However, if the drug is used as a single dose of 10–15 mg/m^2 repeated at 6- to 8-week intervals, mitomycin C may be given safely either as a single agent or in drug combinations. Extreme care must be taken to avoid extravasation of the drug because very significant tissue necrosis will occur.

Although nitrosoureas have some activity against several gastrointestinal cancers (*63*), little data is available on their independent activity against adenocarcinoma of the pancreas. Kovach et al. (*61*) tested 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) against pancreatic carcinoma and found no response in 21 patients. However, the naturally occurring methylnitrosourea streptozotocin does have activity. In their review, Broder and Carter (*64*) found reports of objective responses in 4 of 13 patients (31%). The main toxicities of streptozotocin are nausea, vomiting, and renal toxicity (which may result in renal tubular acidosis, a Fanconi-like syndrome, and azotemia). However, if doses of streptozotocin are kept between 1.0 and 1.5 g given iv weekly, renal toxicity is usually minimal, the nausea and vomiting is tolerable, and the drug may be safely used in patients with pancreatic carcinoma (*65*).

The use of combination chemotherapy in advanced pancreatic cancer has been disappointing. Response rates (*50*) have generally not been any better than the approximate 20% reported with 5-FU alone. Recently, however, Kovach et al. (*61*) reported a prospectively controlled study comparing 5-FU, BCNU, and the combination of 5-FU + BCNU in patients with advanced pancreatic carcinoma. 5-FU and BCNU were given in loading courses whether used singly or in combination. When the combination was used, the total dosages of 5-FU and BCNU were decreased by 20%. Objective responses were observed in 5 of 31 (15.1%) patients receiving 5-FU, 0 of 21 receiving BCNU alone, and 10 of 30 (33.3%) receiving 5-FU + BCNU. Although there were no statistically significant differences in response rate between the combination and 5-FU alone and no differences in patient survival among patients on the three arms of the study, the suggestion of improved response rate with combination chemotherapy is encouraging and should stimulate the development of more combination chemotherapy protocols with the known active agents. An uncontrolled confirmation of the work of Kovach et al. was provided by Lokich et al. (*66*), again with 5-FU and BCNU. In their study, 9 of 15 (60%) evaluable patients had either an objective response (4/15) or stabilization of disease (5/15) after combination chemotherapy with 5-FU + BCNU. The responders had a longer median survival (11 mo) than did the nonresponders (4 mo), though the overall numbers in each group are too small to make this difference significant.

A provocative study with combination chemotherapy in patients with advanced pancreatic carcinoma has been reported by Waddell (*67*). Thirteen patients treated weekly with 5-FU plus daily oral testolactone or testolactone and spironolactone were compared to a retrospective control group that received weekly 5-FU and Coumadin. The patients were well matched for extent of disease and operative procedures. The median survival in the patients given 5-FU and Coumadin was 5 months, whereas the median survival of patients treated with 5-FU and spironolactone had not been reached by 21 months. This difference was statistically significant ($P<0.001$). Why the combination of 5-FU and testolactone would have this striking effect is not clear, though some in vitro (*68*) evidence suggests that testolactone may inhibit purine synthesis. The combination of 5-FU and testolactone is currently being tested in a controlled prospective trial by the Eastern Cooperative Oncology Group, and the results of this study will be very valuable in assessing possible activity of this regimen.

It can be seen that the current status of chemotherapy in advanced pancreatic carcinoma leaves much to be desired. We feel that an approach on two fronts should be useful in developing more effective chemotherapy in this disease. More chemotherapy agents should be taken to phase II clinical trial in an attempt to identify and increase the numbers of drugs with activity against pancreatic carcinoma. This would eventually allow for the development of effective drug combinations, an approach that has been successfully applied in many other tumor categories. Utilizing this principle currently at Georgetown, we are testing a well-tolerated combination chemotherapy regimen with all three known active single agents. The regimen consists of mitomycin C (10 mg/ m^2) every 6 weeks, streptozotocin (1.0 g/m^2) and 5-FU (600 mg/m^2) every week for 2 weeks, then a 2-week rest period.

The physician caring for the patient with pancreatic carcinoma must attempt to manage many difficult symptoms that accompany the disease. As previously noted, pain is a cardinal problem almost universally present. Usual measures including the liberal use of narcotics may frequently be satisfactory; occasionally, patients will continue to have intractable pain despite massive amounts of analgesics. Neurosurgical intervention is worth consideration in carefully selected patients. Cordotomy is not usually efficacious because most patients have bilateral pain, and bilateral percutaneous cervical cordotomies carry the risk of producing respiratory insufficiency. Bilateral thoracic cordotomies or rhizotomies are quite substantial procedures in a poor-risk patient, but percutaneous splanchnic nerve block of the involved thoracic dorsal roots may provide relief.

The wasting and cachexia of patients with pancreatic cancer should be treated with much emotional support and encouragment of the patient to eat. The use of high-protein, high-caloric liquid diet supplements may also be useful. We also routinely use pancreatic enzyme supplementation (Cotazym, Viokase) in patients who persistently lose weight despite adequate caloric intake, whether or not clinical manifestations of malabsorption are present.

In summary, we wish to emphasize again that recent developments have lent a more positive aspect to the management of patients with pancreatic cancer. For many years the patient with pancreatic carcinoma was faced with the specter of having a basically hopeless disease with a very limited prognosis. We have tried to communicate in this discussion that all the manifestations of pancreatic cancer can be treated with some degree of effectiveness. Much active work is proceeding in the diagnosis and treatment of this disease. With an aggressive multidisciplinary approach to the patient with pancreatic carcinoma, the future in the management of this illness may be expected to become somewhat brighter.

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ADDENDUM

Diagnosis

Combinations of diagnostic tests may increase sensitivity and specificity in diagnosing pancreatic cancer. DiMagno et al. (*1*) prospectively tested 70 patients with suspected pancreatic cancer by using the following six procedures: ultrasonography, pancreatic function tests (cholescystokinin-stimulated enzyme outputs), [^{75}Se]methionine scanning, thermography, endoscopic retrograde pancreatography (ERCP), and selective arteriography. Thermography lacked sensitivity and was abnormal in only 15% of patients with pancreatic disease. Pancreatic scanning had an unacceptably high degree of false positives (30%). The combination of ultrasonography, pancreatic function testing, and ERCP was the most sensitive and specific combination for detecting or excluding pancreatic disease. These three tests correctly identified 90% of all patients with pancreatic disease and 80% of the patients with pancreatic cancer.

The pancreatic oncofetal antigen (POA), originally described by Banwo et al. (*2*), has been further characterized by Gelder and co-workers (*3*). POA is a glycoprotein with a molecular weight between 800,000 and 900,000 daltons. It is found in the fetal pancreas and in pancreatic cancer tissue but not in the normal adult pancreas. POA is not specific for pancreatic adenocarcinoma. Gelder et al. (*3*) demonstrated POA in the sera of 20 of 26 (77%) patients with pancreatic carcinoma but also detected the antigen in 2 of 11 (18%) patients with carcinoma of the colon, 3 of 4 (75%) with carcinoma of the biliary tract, and 2 of 12 (17%) with pancreatitis. POA is a tumor-associated rather than a tumor-specific antigen and thus is similar to CEA. Therefore, it is likely that POA will not prove helpful in early diagnosis of pancreatic carcinoma. The usefulness of following levels of POA in monitoring the treatment of patients with pancreatic carcinoma has yet to be defined.

Treatment

A phase I–II trial (*4*) of fast neutron irradiation in patients with locally unresectable pancreatic carcinoma has been completed. Seventeen patients were treated with a total of 1,700 to 1,750 neutron rads to the pancreas for 6 weeks. Five (41%) of the 12 patients with measurable disease responded to therapy. Responding patients had a median survival of 13 months (range 6–18+ mo). Nonresponding patients had a median survival of 4 months (range 2–5 mo). Major toxicities associated with fast neutron irradiation were nausea and vomiting in 66% of patients. Further evaluation of fast neutron irradiation in pancreatic cancer is being done in a national study by the Radiotherapy Oncology Group.

A phase II Chemotherapy Study (*5*) was completed on the combination chemotherapy regimen of streptozotocin, mitomycin-C, and 5-FU (SMF). Twenty-three patients with advanced adenocarcinoma of the pancreas were treated with SMF and 10 of 23 (43%) responded to therapy. The median duration of response was in excess of 7 months. Responding patients lived significantly longer (median, 7.5+ mo) than those with progressive disease (median, 3 mo). The SMF regimen was well tolerated, with the main toxicities being mild myelosuppression and nausea and vomiting. Reversible nephrotoxicity as manifested by proteinurea was seen in 30% of the patients; mild azotemia persisted in 9% of them. The SMF regimen is currently being tested in phase III trial by the Gastrointestinal Tumor Study Group.

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GUEST EDITORIAL by Stephen K. Carter, *M.D.*[1]

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Cancer Treatment Today and Its Impact on Drug Development, With Special Emphasis on the Phase II Clinical Trial

Chemotherapy is one of the five major modalities available for the treatment of cancer. Since the discovery of the antitumor action of nitrogen mustard during the midforties, chemotherapy has developed rapidly to a position where it is involved in a wide range of curative therapies and constitutes a separate subspeciality of medical oncology.

The chemotherapeutic approach met with early success in hematologic malignancies, particularly childhood leukemia (*1–3*) and Hodgkin's disease (*3–6*), and also showed potential activity against solid tumors in trophoblastic neoplasms (*7–9*) and testicular carcinoma (*10, 11*). Studies of pediatric solid tumors provided the first evidence that chemotherapy combined with surgery and/or radiotherapy could markedly enhance the curative potential of these local modalities. This was first observed in Wilms' tumor (*12–14*) and later confirmed in embryonal rhabdomyosarcoma (*15–17*), retinoblastoma (*18*), and Ewing's sarcoma (*19, 20*). Advances in the treatment of pediatric solid tumors have continued in the recent successes achieved by chemotherapy after amputation in osteosarcoma (*21–23*).

The major current thrust of chemotherapy is its integration into a combined modality attack against adult solid tumors that are the major causes of cancer mortality (*24*). The basic hypothesis in this approach is that surgery and radiotherapy fail to cure because undiagnosed tumor cell microfoci existing outside the range of local therapy ultimately produce recurrent metastatic disease. In combined modality therapy, the chemotherapy that can induce objective regression ($>$50% shrinkage of measurable lesions) in recurrent metastatic cancer will be used immediately after local ablative therapy in the hope of eradicating residual tumor dissemination. This approach has gained dramatic success for breast cancer (*25, 26*) and is being pursued vigorously with cautious optimism about the future for cancer of the colon and rectum (*27*), stomach (*28*), and ovaries (*29*).

The progress made in chemotherapy over the past 30 years and its changing role in the armamentarium of clinical oncology have major implications for the future direction of drug development programs.

The final common pathway of modality-oriented development is being increasingly recognized as disease-oriented therapy. For each specific tumor type, at each stage of the disease, surgery offers a range of radical excisions, radiotherapy provides a number of fractionation schedules and new delivery approaches, and medical oncology affords a wide range of new and old drugs with myriad possibilities for combinations. More recently, immunologic techniques are being used to enhance host immune response to tumor-specific antigens. With this variety of therapeutic tools available, it is not surprising that the practitioners of each modality believe that their particular questions are of critical importance. However, in clinical trials the "id" of modality-oriented development must be tempered by the "superego" of disease-oriented strategy to attain the "ego" of maximum therapeutic success.

With the new emphasis on combined modality therapy and a disease-oriented approach, the problems of conducting clinical trials have become especially acute in the field of chemotherapy. The patient population currently available for evaluation of new drugs consists of individuals with more advanced disease and a history of more prior therapy, whereas the criteria for measurement of response to treatment have become more stringent. These conditions have their greatest impact in the sector of drug development that is seeking analogues of established active drugs, and this area needs to be seriously reevaluated. In this paper I will discuss the implications of all these factors for the Phase II clinical trial, which is the most important study that determines specific antitumor activity and is the critical interacting point between the modality- and disease-oriented approaches.

CLINICAL TRIALS

The Division of Cancer Treatment in the National Cancer Institute conducts clinical trials of new antitumor agents in three phases specifically modified from those required by the Food and Drug Administration (*30*).

Phase I trials involve clinical pharmacology in patients with tumors resistant to all currently available treatment. The principal aims of this phase of clinical evaluation are to determine the MTD at the schedule tested,

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ABBREVIATIONS USED: MTD=maximum tolerated dose; 5-FU=5-fluorouracil; CCNU=1,3-cis(2-chloroethyl)-1-nitrosourea; DTIC=dimethyltriazenoimidazole carboxamide.

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

establish the parameters of toxicity, and determine whether toxicity is predictable, treatable, and/or reversible. The patients have normal organ function and have not received prior therapy to an extent that would compromise evaluation of toxicology. Their estimated survival is usually 2 months, during which toxicity and response are completely evaluated. Measurable or evaluable disease is not required and a favorable response, though encouraging and significant when it occurs, is not essential to the decision for proceeding with Phase II trials.

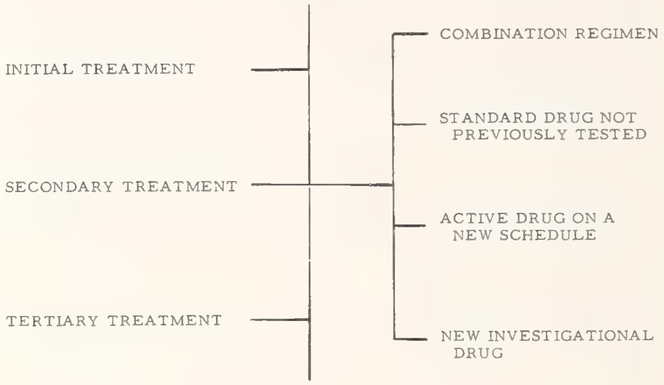
Phase II investigations encompass the "screening" of new drugs to discover clinical antitumor activity worthy of further evaluation. These trials are not designed to provide definitive answers on the ultimate value or function of a given drug, which is the purpose of the larger scale Phase III studies.

The efficacy of a potential anticancer drug must be examined in as many aspects as possible. It is not simply a question of whether the drug is effective, but to what degree it is effective compared with other agents used to treat a given tumor. The estimated MTD derived in Phase I studies is usually chosen to test the efficacy of the drug, and most trials are conducted at only one dose and schedule. Unfortunately, certainty that this dose schedule is anywhere near the optimum for therapeutic effect is impossible. Since studies with animals clearly show that the therapeutic efficacy of many active drugs depends greatly on the dose schedule, in the absence of comparative pharmacology for animals and man different doses and schedules should be used in Phase II trials. However, this approach is rarely feasible.

At the completion of Phase II studies, a reasonable judgment should be possible regarding the degree of efficacy and nature of adverse effects at a particular dose schedule. Consideration of these factors, which can be summed up in the concept of the risk–benefit ratio, determines whether or not further large-scale studies should be conducted. When efficacy is considered the critical point, Gehan and Schneiderman (*31*) have noted that the decision to be reached is whether the agent could be effective in x% of patients or more, or is unlikely to be effective in x% of patients or more. An answer can usually be obtained after the study of a small number of patients.

For many years the Division of Cancer Treatment has used a screening panel of tumors known as signal tumor types. Adenocarcinoma of the breast, colon, pancreas, and ovary, and bronchogenic carcinoma are used to screen for drug activity against the small-growth fraction of solid tumors that are traditionally most resistant to chemotherapeutic attack. The acute leukemias and lymphomas were chosen to represent the rapidly growing and more chemotherapy-sensitive tumor types. This disease-oriented Phase II trial is believed to be superior to the often used general Phase II trial that employs a wide range of tumor types but includes only a few of these with patient numbers large enough to give meaningful results. Commonly in the older literature, a drug is described as inactive on the basis of a low response rate in about 50 patients, of whom only 2 or 3 have colon or breast cancer.

In cancer treatment today, a Phase II investigation of a new anticancer drug is only one of a group of options available to any given population with advanced disease (text-fig. 1). Competing at each time point are combination regimens, standard drugs not previously tested, and known active drugs on new schedules. In addition, combination chemotherapy is so successful against many tumors that a question can be raised on the ethics of testing a new drug in patients who have not been previously treated by chemotherapy. A list of such tumors is shown in table 1A. These tumors are obviously the most responsive to chemotherapy. Therefore, a new drug entering clinical study today will face a situation significantly different than in 1955. Twenty years ago a new drug was evaluated in new patients, and any reasonable degree of activity was acceptable. Today the same drug would be used in patients with advanced disease who have been heavily pretreated and have compromised



TEXT-FIGURE 1.—Therapeutic options in advanced disease.

TABLE 1.—*Suitability of tumor types for Phase II trials*

A) Tumors in which "standard" treatment is now usually administered before Phase II studies
  Acute leukemias
  Malignant lymphomas
  Chronic leukemias
  Multiple myeloma
  Breast cancer
  Ovarian cancer
  Soft tissue and bone sarcomas
  Testicular carcinoma
B) Tumors in which an argument can be made for "standard" treatment before Phase II studies
  Large-bowel carcinoma
  Head and neck cancer
  Brain tumors
  Stomach cancer
  Uterine cervix carcinoma
C) Tumors in which Phase II studies of new drugs are feasible in new patients
  Bronchogenic carcinoma
  Pancreatic adenocarcinoma
  Malignant melanoma
  Prostate adenocarcinoma
  Bladder carcinoma
  Endometrial carcinoma
  Renal carcinoma
  Esophageal carcinoma

marrows, and the criteria for labeling the drug "active" would require a higher level of antitumor response. In the face of this reality, any drug development program that does not modify the criteria for moving a drug into clinical trial, as well as the clinical trial strategy, will risk overloading the clinic with poorly evaluated drugs resulting in a high number of inactives.

Table 1B lists the tumors for which a case can be made against further Phase II trials in new patients. These tumors will probably soon be added to those in table 1A.

Still another group of tumors for which there is no difficulty in contemplating Phase II trials in new patients (table 1C) has two categories. In some of these tumors chemotherapy generally has been poorly evaluated, and the priority assigned to testing untried standard agents would be higher than that for new agents being investigated. For example, NCI-supported groups are now conducting Phase II trials with endometrial cancer using adriamycin and cyclophosphamide rather than new investigational drugs that have just completed Phase I study. The second category includes tumors such as squamous cell lung cancer, malignant melanoma, and pancreatic cancer, which have been the most unresponsive to chemotherapy. New active drugs are desperately needed for these tumors, and all experimental agents should have a high priority for trial. However, it should be recognized that a drug development program in which only old cytotoxicity screening systems such as L1210 are used will have a high failure rate if it depends only on the second category of tumors for its criteria of success. Intensive work needs to be done to understand why drugs have failed against these tumors and how these tumors differ from responsive ones, so that screening systems can be developed to select drugs with a greater probability of activity.

Some thoughts on clinical trials in specific disease areas will be outlined in the following sections. The data cited on single agent activity are largely from a recently published analysis of the clinical chemotherapy of solid tumors (*32*).

Breast Cancer

Breast cancer is responsive to a wide number of chemotherapeutic agents. Thirteen drugs have shown evidence of activity (table 2), and response rates of 35% are not uncommon for single agents (*33*, *34*). Combination chemotherapy has an inducing ability superior to single agents (*35*). Response rates between 50 and 60% are routinely observed in clinical trial situations with combinations such as Cytoxan, methotrexate, and 5-FU, or Cytoxan, methotrexate, 5-FU, vincristine, and prednisone, and with a variety of combinations involving adriamycin and Cytoxan.

All previously untreated patients with recurrent or inoperable breast cancer amenable to chemotherapy are now placed on a combination regimen or some standard chemotherapy regimen before a Phase II trial can be undertaken. In many instances sequential combination regimens are being used in clinical trials; therefore, these patients will be exposed to six or more drugs before becoming available for Phase II study. In order to be considered for Phase III evaluation, a new compound will have to produce at least a 20–25% response rate with reasonable therapeutic index and potential for combination. A new drug with a 20% response rate and marked marrow toxicity might not enter Phase III study at this time. However, a drug devoid of marrow toxicity would be of great clinical interest. Since adjuvant study could potentially occur with any active drug, careful attention must be given to chronic toxic effects. For example, the cardiac toxicity of adriamycin should give pause to those initiating adjuvant trials with this compound when other active drugs are available that lack such significant potential for chronic toxicity.

TABLE 2.—*Drugs with some evidence of activity against breast cancer*

Drugs	Number of responses/No. of evaluable patients	Response rate %
Cyclophosphamide	182/529	34
Melphalan	20/86	23
5-FU	324/1,263	26
Methotrexate	120/356	34
Vincristine	47/226	20
Vinblastine	19/95	20
Adriamycin	67/193	35
Mitomycin C	41/110	37
Mechlorethamine	32/92	35
Chlorambucil	11/54	20
Carmustine	16/76	21
Hexamethylmelamine	11/54	20
Dibromodulcitol	20/53	37

The use of drug combinations causes difficulties in the clinical evaluation of analogues. How is one to study a new alkylating agent or fluorinated pyrimidine against breast cancer? Only an analogue postulated to be without cross-resistance to its parent compound could be feasibly tested after patients have received combination exposure. Substitution of the new analogue for the parent compound in the combination has been suggested as a possible trial technique. This approach ignores the possibility of pharmacologic interactions and, to show a meaningful difference (unless quite large), such a trial would require patient numbers approaching Phase III proportions.

Lung Cancer

Lung cancer cannot be viewed as a single disease entity for clinical trials. Differences in cell type are clearly reflected in individual clinical course patterns and responses to therapy (*36*). Small-cell anaplastic carcinoma (oat-cell) is responsive to eight drugs on the basis of objective regression criteria (table 3) (*37*). However, the response rates, which are comparable to those seen for breast cancer, result in only minimal improvement in survival.

Combination chemotherapy is now routinely used in clinical trials. Cytoxan is an integral part of all these combinations, and among the most studied are ones that add a nitrosourea (mostly CCNU) with or without methotrexate and ones that add adriamycin plus other drugs such as DTIC. All of these combinations produce re-

TABLE 3.—*Drug activity against lung cancer (small-cell type)*

Drugs	Number of responses/No. of evaluable patients	Response rate %
Cyclophosphamide	92/296	31
Mechlorethamine	31/80	39
Methotrexate	31/79	39
Hexamethylmelamine	24/67	36
Procarbazine	11/44	25
Adriamycin	12/48	25
Carmustine	4/19	21
Lomustine	7/48	15
Methyl-CCNU	4/33	12
Dibromodulcitol	3/15	20

TABLE 4.—*Drug activity against lung cancer (squamous cell type)*

Drugs	Number of responses/No. of evaluable patients	Response rate %
Cyclophosphamide	46/232	20
Mechlorethamine	38/183	21
Methotrexate	35/140	25
Dibromodulcitol	9/39	23
Adriamycin	13/70	19
Bleomycin	34/265	13
Lomustine	10/58	17
Methyl-CCNU	13/80	16
Hexamethylmelamine	11/99	11
Procarbazine	7/55	13

sponse rates of 50% or greater. Although survival gain is observed with these combinations, it is not dramatic and does not reflect the magnitude such a response rate would lead one to expect.

Phase II testing in most clinical trial groups would be relegated to patients who have failed combination regimens. Since survival is usually short after the failure of chemotherapy and the performance status is generally poor, this is a difficult group of patients in which to study new drugs. Such a situation is unfortunate because new drugs are greatly needed in this area, and the most aggressive drug combinations plus radiotherapy are not achieving any real gains in long-term disease-free survival. On the basis of past experience, a new active drug would have to induce a response rate of at least 20% to be clinically interesting and, ideally, it should be higher for the drug to have an impact in combinations. Pharmacologically, a drug that can cross the so-called blood–brain barrier would be of great interest, since small-cell anaplastic disease often is manifest in the central nervous system. Analogue testing would encounter all the difficulties described earlier for breast cancer.

For the other cell types, single-agent chemotherapy is almost completely ineffective. Although a variety of drugs produce some response (table 4) (*37*), their impact on survival is nonexistent. In some instances, combinations have increased response rates and made some impact on survival, but no data exists to indicate that combinations require initial priority. A new drug could be tested in new patients with advanced disease, and in many groups this is commonly done. For a drug to be interesting, it would have to show more than a 20% response rate with no survival gain. Past experience has shown that the aggressive combination of such drugs is probably not going to have a major effect on survival. Single drugs that can prolong survival are needed, and survival should be one of the major end points for Phase II studies of this group of lesions.

Large-Bowel Cancer

Only six drugs are reported to have any activity against large-bowel cancer (table 5) (*27*). Of these, only 5-FU, mitomycin C, and the nitrosoureas (especially methyl-CCNU) are meaningfully active. The 21% response rate with 5-FU is the highest reported; this tumor must be viewed as poorly responsive to single agents.

TABLE 5.—*Drug activity against colon cancer*

Drugs	Number of responses/No. of evaluable patients	Response rate %
5-FU	454/2,107	21
Mitomycin C	35/218	16
Methyl-CCNU[a]	18/148	12
Cyclophosphamide[a]	15/71	21
Methotrexate[a]	19/111	17
Carmustine	17/128	13
Hexamethylmelamine[a]	11/95	12

[a] Small numbers of patients from non-disease-oriented studies.

The combination of methyl-CCNU and 5-FU with or without vincristine has given response rates between 30 and 43% (*38, 39*). In most clinical trial groups, untreated patients are being entered in combination studies, and current Phase II studies in these groups would be conducted in patients failing on these combinations or not eligible because of prior 5-FU exposure. The Mayo Clinic group has shown that the survival of 5-FU failures without further treatment is less than 3 months; this is another poor-risk group for new drug testing. If the current studies with combinations of the few active drugs do not show meaningful survival impact, the untreated patients might return as a Phase II resource. New drugs are obviously needed, and any new structure with an activity of 20% would be of interest for use in combinations. Analogues of any of the active structures could easily be tested.

For large-bowel cancer, a Phase II study design randomizing half the patients to a combination of drugs and half to a new drug could be used. A crossover would be made at the point of recurrent disease, so that both groups would have potential exposure to both the combination and the new drug. This approach is attractive because it keeps some new patients available for Phase II study but denies no patient the combination approach.

Stomach Cancer

Stomach cancer has been neglected in Phase II study in the United States. Until recently, a few patients with stomach cancer were included in large-bowel cancer protocols and only fragmentary data resulted. Only two drugs are clearly active (table 6) (*40*), but most standard

TABLE 6.—*Drug activity against stomach cancer*

Drugs	Number of responses/No. of evaluable patients	Response rate %
5-FU	101/448	23
Mitomycin C	63/211	30
Mechlorethamine[a]	4/25	16
Chlorambucil[a]	3/18	17
Adriamycin[a]	7/27	26
Carmustine	6/33	18

[a] Small numbers of patients from non-disease-oriented studies.

agents still must be adequately evaluated and new experimental drugs might have to take a secondary position on the priority scale.

Combinations of nitrosoureas plus 5-FU are active against this tumor and yield response rates exceeding 40% (*41*). If the response rates for combinations continue to be in this range, with resultant survival gain, Phase II studies will soon be forced into a secondary role.

Genitourinary Tumors (Excluding Testicular Cancer)

Cancer of the prostate, bladder, and kidney have been neglected areas in Phase II evaluation of new anticancer drugs. The small volume of data has been gathered in broad studies of solid tumors including a few genitourinary cancers and/or far-advanced prostate and kidney tumors after failure of hormonal evaluations (*42*). At this time there is no way to measure the intrinsic responsiveness of these tumors to chemotherapy.

The highest priority must be given to Phase II evaluation of standard agents before the large backlog of new investigational drugs can be approached. The problem of using new patients for Phase II studies is that many urologists believe that estrogens must be used initially against prostate lesions and, similarly, that progestogens must be used against renal carcinoma. Unfortunately, patients who fail to respond to these hormones often have extensive disease and are poor candidates for further evaluation. One approach that could be undertaken would be a randomization between Phase II drug and hormone therapy with appropriate cross-over. This would allow evaluation of new drugs in some new patients and not deny any patient a chance for response to the hormone therapy.

Testicular Carcinoma

Testicular carcinoma is highly responsive to single-agent chemotherapy (table 7) (*42*), which is curative in a small percentage of patients. Newer combinations based on vinblastine plus bleomycin are increasing the curative potential in advanced disease (*43–46*). Carcinoma of the testes is a rare tumor, and breakdowns by cell type make it analogous to lung cancer in the need for therapeutic stratification.

Phase II testing in most groups will be relegated to individuals that fail to respond to combination studies, and only small numbers of patients can be expected. Drugs for Phase II testing will have to be carefully selected. A reasonable approach would be to consider only drugs with established evidence of activity against some other type of cancer. This tumor will also be a poor one for analogue testing. To achieve Phase III evaluation, a new drug will have to induce at least a 40% response rate and have a potential for combination usage.

Malignant Melanoma

Only one drug (DTIC) is clearly active against malignant melanoma, and the overall response rate of 21% makes this tumor one that is least responsive to chemotherapy (*47*). Combinations have been extensively tested, but none have been more effective than single agents. New drugs are badly needed, and Phase II studies may be readily performed among new patients.

The major conflict among clinical trials in advanced disease arises from the fact that immunotherapy is being widely studied in patients with melanoma. Therefore, a new drug active against melanoma should also be compatible for use in combination with some type of immunostimulation.

Ovarian Cancer

Alkylating agents used alone give objective regression in approximately 50% of individuals with ovarian cancer. Like testicular cancer, neoplasms of the ovary are among the solid tumors most responsive to single agents. In addition to alkylating agents, a variety of other drugs shows evidence of activity (table 8) (*48*), but many standard drugs still remain to be evaluated. Combinations are now being vigorously studied in many centers, yet none have produced results consistently superior to single agents.

Many Phase II trials are being performed in patients with recurrent disease; thus new active drugs will be

TABLE 7.—*Drug activity against testicular cancer*

Drugs	Number of responses/No. of evaluable patients	Response rate %
Vinblastine	17/25	52
Actinomycin D	16/31	52
Mithramycin	155/431	36
Bleomycin	24/57	42
Melphalan	49/86	57
Adriamycin	12/60	20

TABLE 8.—*Drug activity against ovarian cancer*

Drugs	Number of responses/No. of evaluable patients	Response rate %
Cyclophosphamide	115/262	44
Chlorambucil	200/390	51
Melphalan	235/494	47
5-FU	32/101	32
Adriamycin	18/48	38
Hexamethylmelamine	21/54	39
Mechlorethamine	25/81	31
Lomustine	7/22	32
Methotrexate	4/16	25

difficult to detect because response rates in chemotherapy failures are significantly lower than in new patients. The M. D. Anderson Hospital and Tumor Institute has recently compared melphalan versus hexamethylmelamine and adriamycin in previously untreated patients with the use of the controlled crossover techniques discussed earlier. Both drugs were highly active. This kind of trial was easily undertaken because preliminary evidence existed for the activity of both test drugs. Whether such a trial would be undertaken with a new drug just completing Phase I study is doubtful.

The tools exist for successful combination chemotherapy against ovarian cancer, and combinations soon will become standard initial therapy as in other highly responsive tumors. New drugs will have to produce a 25–30% response rate against recurrent advanced disease to be seriously considered for further study. The drugs will also have to be easily combined and chronic toxicities will be important, since the combined modality approach to early disease is now under study by clinical trial groups.

Cancer of the Uterus

Carcinoma of the uterine cervix (predominantly squamous cell disease) and corpus (predominantly adenocarcinoma) have been neglected in disease-oriented Phase II trials. Cancer of the uterine cervix appears to be responsive, with nine drugs showing some evidence of activity (table 9) (*29, 32*). However, data obtained from more than 100 patients are available for only two drugs. The two highest priorities for clinical study are 1) a full determination of the effectiveness of the drugs that show preliminary evidence of activity, and 2) the testing of standard agents for which no data are available. In addition, the potential tools exist for combination regimens; these should be considered in the priorities for clinical material. Phase II testing of newer drugs would probably be given low priority at this time and be performed only in patients failing on some primary regimen.

No active nonhormonal drugs exist for endometrial cancer (*49*), but none have been adequately studied and Phase II trials need to be initiated. A critical question in planning these studies will be the requirement for initial therapy with progestational agents. This hormonal approach does work in approximately 30% of cases, with many of the remissions lasting for a significant time. This is another tumor for which the controlled crossover Phase II approach could be contemplated.

TABLE 9.—*Drug activity against cervical cancer*

Drugs	Number of responses/No. of evaluable patients	Response rate %
Cyclophosphamide	36/187	19
5-FU	37/159	23
Chlorambucil	11/44	25
Melphalan	4/20	20
Methotrexate	5/24	21
Vincristine	10/34	29
Adriamycin	9/28	32
Bleomycin	8/78	10
Methyl-CCNU	4/24	17
Hexamethylmelamine	11/40	28

Central Nervous System Tumors

Tumors of the brain present many problems for Phase II study. These include the pharmacologic sanctuary of the blood–brain barrier, a heterogeneity of pathologic subtypes, small numbers of patients available for study, and the difficulty of objective measurement of response.

Chemotherapy studies have been done mainly with the malignant gliomas. A range of drugs shows evidence of activity (table 10), but only the nitrosoureas and procarbazine are clearly active (*50–52*). A question that remains to be resolved is whether a drug must cross the intact blood–brain barrier to be possibly active. Since the blood–brain barrier appears to be broken when a tumor is present, this pharmacologic characteristic may not be required for an active drug. Generally, however, the active drugs all can penetrate the central nervous system, and several drugs lacking this property have not shown meaningful activity. Because of the few patients available, Phase II testing probably should involve predominantly drugs that do penetrate as evidenced by either pharmacokinetic study or activity against intracerebrally implanted experimental tumors.

An argument can be made for treatment of all patients with nitrosoureas before a new drug study is initiated, but it lacks full persuasiveness since the survival is still short in patients with advanced disease treated with nitrosoureas.

Head and Neck Cancer

Squamous cell lesions of the head and neck comprise tumors in various sites ranging from the lip to the hypopharynx. They have a range of survival potential based on stage and site after primary therapy with either surgery or radiotherapy. Whether these sites also exhibit different patterns of response to chemotherapy has never been clarified because clinical data, especially for individual site, are lacking. Most studies simply include all head and neck tumors together. Despite the potential heterogeneity, head and neck lesions appear to be responsive to chemotherapy (table 11) (*53, 54*). Methotrexate administered by a range of schedules induces responses in nearly half the patients. The tools exist for combination regimens, and these studies should be pursued with a higher priority than Phase II

TABLE 10.—*Drug activity against brain cancer*

Drugs	Number of responses/No. of evaluable patients	Response rate %
Carmustine	67/142	47
Lomustine	47/115	41
Methyl-CCNU	13/40	33
Methotrexate[a]	35/65	54
Vincristine[a]	33/58	57
Vinblastine[a]	21/48	44
Procarbazine[a]	14/27	52

[a] Small numbers of patients from non-disease-oriented studies.

TABLE 11.—*Drug activity against head and neck cancers (all types)*

Drugs	Number of responses/No. of evaluable patients	Response rate %
Methotrexate	298/630	47
Bleomycin	24/158	15
Cyclophosphamide[a]	28/77	36
Chlorambucil[a]	5/34	15
5-FU[a]	18/118	15
6-Mercaptopurine[a]	6/45	12
Vinblastine[a]	10/35	29
Adriamycin[a]	13/67	19
Methyl-CCNU[a]	6/40	15
Hexamethylmelamine[a]	9/75	12
Dibromodulcitol[a]	11/50	22
Hydroxyurea[a]	7/18	39

[a] Small numbers of patients from non-disease-oriented studies.

evaluations of new agents. In addition, adjuvant studies are beginning to be developed. Phase II trials could still be undertaken in new patients, but this situation will probably not persist for a very long time.

Pancreatic Cancer

Pancreatic cancer has a dismal prognosis regardless of the therapy used (*55*). Patients with disseminated disease have an extremely short survival and many are in a poor-risk category. In addition, measurable lesions are often not present. Since survival is so short, it is an excellent end point and, like squamous lung cancer, objective regression rates of 20% without survival gain will probably not provide the building blocks for successful combination approaches. Only two drugs have shown any evidence of response (5-FU and mitomycin C), and many standard drugs have not been tested. Combinations have all failed to change the grim picture. New drugs can easily be studied in patients with newly diagnosed disease, but survival must be the end point in clinical studies designed to meet this need.

Malignant Lymphomas

The malignant lymphomas are highly responsive to chemotherapy, and a curative potential exists when drugs are optimally used (*56*). The treatment flow in advanced disease is prolonged combination treatment. Patients who do not respond to this approach may be treated by a second combination or by any of a number of active single agents not present in the primary combination.

Phase II trials should be reserved for patients with multiple recurrence. Such patients will probably have compromised bone marrow reserve and be in a poor-risk category. Phase II trials can no longer be reasonably undertaken with all new drugs completing Phase I study. A careful selection of drugs will have to be made on the basis of characteristics—i.e., pharmacologic distribution, toxicity attributes (acute and chronic), postulated mechanism of action, and evidence of clinical activity against other tumors. A drug should produce at least a 30–40% response rate to be considered for further study unless it does not cause marrow toxicity.

The malignant lymphomas are particularly difficult tumors for analogue testing. The standard active agents are involved in combination chemotherapy studies of curative intent, with the major end point being disease-free remission and survival as well as induction. These trials now take years for completion and evaluation. How a new alkylating agent or *Vinca* drug can be tested at this time against these tumors is difficult to visualize. Treatment of recurrent disease would be unreasonable, and the integration of an analogue into a primary combination would not only be risky but also would require years of study to achieve a Phase II answer.

Adult Acute Leukemia

Adult leukemia is predominantly myeloblastic leukemia. Lymphoid leukemia in adults does not have the same degree of responsiveness as it does in children. Adult leukemia responds to several drugs, and combinations can induce complete remission in 50–70% of patients with newly diagnosed disease (*57*). As induction has become more successful, emphasis has switched to consolidation and maintenance regimens that may include immunotherapy as well as chemotherapy. New drugs are still needed for this disease, but Phase II trials can no longer be performed in new patients. Recurrent disease will probably be more difficult to treat; however, prior responders are a select group of more responsive patients because those who fail initial therapy often expire during induction treatment.

Acute Leukemia of Childhood

In childhood leukemia, long-term disease-free survival is the end point of any therapeutic trial against primary disease. Clinical studies must be designed to consider the exigencies of extended follow-up and the years that must pass before the therapeutic questions can be answered. For this reason clinical evaluation of an analogue poses great strategic difficulties.

Although analogues of vincristine, methotrexate, and 6-mercaptopurine continue to be developed and placed into clinical trial, it is hard to determine how they will undergo Phase II evaluation in this disease. New structures face an ever diminishing test population because more and more children are being entered in highly sophisticated treatment and support regimens that are leading to increased disease control. Only a limited number of carefully selected compounds can reach Phase II evaluation. The criteria that should govern their selection include 1) activity against other tumor types, 2) ability to enter a pharmacologic sanctuary, 3) favorable toxicologic characteristics (e.g., lack of marrow toxicity), 4) potential ease of combination with other agents, and 5) consideration of the potential for chronic toxicity.

DISCUSSION

When anticancer drug development began over 20 years ago, cancer therapy was vastly different than it is today. Surgery and radiotherapy were the major modalities of therapy. With the exception of trophoblastic neoplasms, chemotherapy could accomplish little except occasional short-lived responses without impact on survival. At the time, the need for a new modality was clearly evident and a large number of drugs were put into clinical trial. Any drug therapy that worked was exciting, and the clinicians who pioneered in the field of medical oncology had the thrill of watching certain tumors become responsive to drug treatment and of observing meaningful survival gains that held a reasonable expectation of cure. The current techniques of drug development are largely the same ones that were established in the 1950's. Although these methods have become highly refined, they have not been significantly modified. However, cancer treatment has changed, and it is time for those involved in drug development to reevaluate their approaches in the light of the changed perspective.

Five major changes in cancer treatment that have an impact on drug development are listed in table 12 with some of their implications for drug development.

At present, 31 nonhormonal anticancer agents (table 13) have activity against at least one type of human cancer. This means that the potential for analogue development is now infinitely greater than it was 20 years ago. We have to ask ourselves what the balance should be between analogue development and the search for new structures. We must question whether the same systems that discovered the parent compound will be the correct systems in which to detect a superior analogue. The clinical needs for individual tumor types must be studied in relation to the feasibility of testing analogues, new structures, or both. The very fact that so many active structures are available may mean that our criteria for selecting new structures for clinical trial must be more stringent. This problem is compounded by the possibility that it may be more difficult for the old predictive systems to find new structures. The many active drugs also raise clinical expectations for what a new drug should accomplish.

Combination chemotherapy has been the approach that has led to the major curative advances with chemotherapy against such diseases as childhood leukemia and Hodgkin's disease. The present success of combination approaches means that, in the drug development area, greater attention must be given to the discovery of active drugs with pharmacologic and toxicologic characteristics enabling integration into combination regimens. The combination approach also restricts clinical testing of analogues against the most responsive tumors, as discussed in greater detail in the sections on specific diseases. Since combinations are producing such high response rates in so many tumors, the expectation for the clinical activity level of a new drug will be higher in a situation in which only previously treated patients will be available for study. The combined modality approach, with its emphasis on long-term adjuvant chemo-

Table 12.—*Changes in cancer therapy and implications for drug development*

Change	Implications for drug development
I. Discovery of many active structures	A) Increased potential for analogue development B) More difficulty in finding new structures C) Greater clinical expectation D) More difficulty in clinical evaluation
II. Widespread applications of combination chemotherapy	A) Loss of most responsive tumors (e.g., new patients) for optimal clinical evaluation B) Increased emphasis on toxicologic and pharmacologic considerations C) Greater difficulty for analogue testing D) Greater clinical requirement for "active" designation
III. Combined modality approach and IV. Long-term adjuvant chemotherapy	A) Possibility of significant diminishment in the role of chemotherapy in advanced disease B) Increased emphasis on problems of chronic toxicity C) Requirement for new model systems D) New parameters for evaluation E) Difficulty for analogue testing
V. Rise of immunology and immunotherapy	A) Competition for cytotoxic drugs B) New parameters for evaluation (e.g., immunosuppression) C) Requirement for new model systems

Table 13.—*Nonhormonal agents active in at least one type of cancer*

Classification	Drug
Alkylating agents	Cyclophosphamide Mechlorethamine Chlorambucil Melphalan Busulfan
Antimetabolites	5-FU Methotrexate 6-Mercaptopurine 6-Thioguanine Cytosine arabinoside
Mitotic inhibitors	Vincristine Vinblastine
Antitumor antibiotics	Actinomycin D Mithramycin Daunorubicin Adriamycin Bleomycin Mitomycin C
Random synthetics and miscellaneous	BCNU[a] CCNU Methyl-CCNU Streptozotocin DTIC Hexamethylmelamine Dibromodulcitol Hydroxyurea Procarbazine L-Asparaginase Dibromomannitol 5-Azacytidine

[a] BCNU=1,3-bis(2-chloroethyl)-1-nitrosourea.

therapy, places increased emphasis on the problem of chronic toxicity. A successful drug will have to be potentially capable of administration for prolonged periods in patients with a cure expectation. Drugs with chronic toxic effects will be less attractive in this area.

As a result of therapeutic success, another potential difficulty of drug development is the long-range possibility that, as disease control is increased, the role of chemotherapy against advanced disease will be significantly diminished. As cancer therapy and diagnostic acumen become more successful, advanced disseminated disease will no longer be commonplace; therefore, the classic testing ground for new drugs will shrink. Ultimately, if drug development is completely successful, it will "put itself out of business."

The rise of immunology and immunotherapy in the armamentarium of therapy contributes another important new factor that reverberates in the drug development milieu. The new immunotherapeutic tools are another competitive element for existing clinical resources. In addition, the immunosuppressive potential of cytotoxic drugs is an important clinical parameter that must be considered in new drug evaluation.

In conclusion, the medicinal chemists and experimental biologists involved in drug development need to reevaluate their approaches and concepts in light of the changing picture of cancer treatment. Chemotherapy is one of the major causes of optimism in cancer therapy research today. The scientists who have worked in the field have a right to be justifiably proud of their accomplishments. However, no scientific area is static, and some change of approach in drug development will be an important task for the immediate future.

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ADDENDUM

The phase II trial still continues to be an area of great importance and great difficulty. The continued success of cancer chemotherapy continues to make phase II evaluation of new drugs more difficult. Fewer and fewer patients, without prior exposure to therapy, are now ethically available to phase II study with new drugs. When this is true, the most unresponsive tumor types become involved in cancer chemotherapy. The more intrinsically responsive the tumor type to drug treatment, the more heavily pretreated must be the potential phase II study population.

Analogs pose a specific problem above and beyond those problems intrinsic to all phase II studies. When the parent compound is successful, it is usually part of an aggressive combination regimen. If one uses the analog after exposure to a combination containing the parent, then the test is not a fair one, unless lack of cross-resistance is postulated. Substitution of the analog for the parent within a combination is a possibility, but this runs the risk of pharmacologic interactions hiding a potentially positive effect. In addition, this type of substitution requires a phase III trial design and numbers to answer a phase II trial.

At this time, phase II trials could be reasonably undertaken with a new drug in the following tumor types without prior exposure to drugs: 1) Non-oat cell bronchogenic carcinoma, 2) large bowel cancer, 3) adenocarcinoma of the pancreas, 4) malignant melanoma, 5) prostrate adenocarcinoma, 6) endometrial carcinoma, 7) renal carcinoma, and 8) esophageal carcinoma.

Tumors for which an argument can now be made for "standard treatment" before phase II studies include: 1) head and neck cancer, 2) brain tumors, 3) stomach cancer, 4) uterine cervix carcinoma, and 5) bladder carcinoma.

The tumor types in which standard treatment is usually administered before phase II studies is unchanged from table 1 in the Editorial.

GUEST EDITORIAL

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Adjuvant Chemotherapy of Osteogenic Sarcoma: Progress and Perspectives[4]

In July 1972, adjuvant chemotherapy studies in OGS were initiated at the Sidney Farber Cancer Institute-Children's Hospital Medical Center. The following is an analysis of our experience to date with consideration of some of the problems, interpretations, and perspectives.

It is increasingly in the nature of clinical therapeutic research in oncology that the definitive evaluation of a new treatment program requires a lengthy follow-up period—often several years. This is particularly true of adjuvant studies generally and of adjuvant chemotherapy studies in OGS particularly. Thus, in the process of performing these studies in OGS, we have been continuously studying and planning means by which we may increase the effectiveness of current adjuvant programs. Such studies are built on a preliminary interpretation of ongoing studies. In keeping with this dilemma, this editorial will consider 1) a critical retrospective evaluation and interpretation of what, in fact, has been accomplished and 2) what future directions of adjuvant chemotherapy in OGS might be taken.

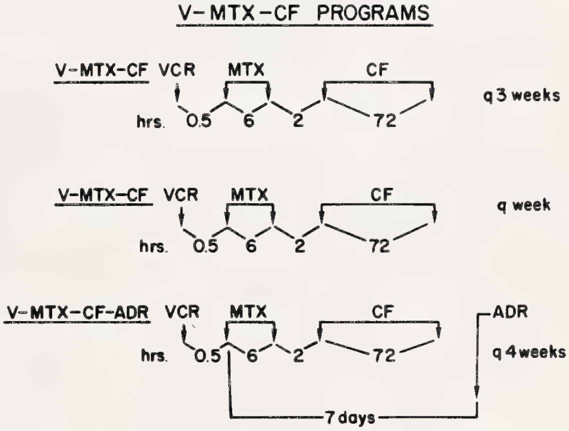
The application of chemotherapy as an adjuvant for a given category of neoplastic disease depends on the development of effective chemotherapy programs for overt macrometastatic disease (table 1). Prior to 1970, the only agents of probable but limited activity in overt OGS were the alkylating agents, with which response rates up to 20% were reported (*1*). In the early 1970's, adriamycin was found to be effective in 30–40% of such patients (*2*). At about the same time, Jaffe (*3*) and Djerassi (*4*), using high-dose MTX-CF programs, demonstrated a similar response rate. MTX, because it is myelosuppressive, has been used intermittently at 3-week intervals to allow for recovery from myelosuppression. However, with proper monitoring of the pharmacology of MTX and of renal function and with the use of fluids and alkali, myelosuppression with high-dose MTX-CF occurs infrequently (*5*), and it has been possible to deliver this program on a weekly basis (text-fig. 1). Consistent with the experimental evidence for a steep dose-response curve for MTX, this program has produced an objective response in over 80% of patients with overt OGS (*6*).

TABLE 1.—*Macrometastatic OGS: Effectiveness of chemotherapeutic agents*

Agents	Objective response[a] %
Alkylating agents	20
Adriamycin	35
MTX-CF every 3 wk	35
MTX-CF every wk	80

[a] Greater than 50% decrease in product of two diameters.

The first adjuvant program for OGS involved high-dose MTX-CF every 3 weeks (text-fig. 1). Vincristine



TEXT-FIGURE 1.—OGS: High-dose MTX treatment programs. V and VCR=vincristine; ADR=adriamycin.

ABBREVIATIONS USED: OGS = osteogenic sarcoma; MTX-CF = methotrexate (MTX)-citrovorum factor; NED = no clinical or roentgenographic evidence of disease; Ara-C = cytosine arabinoside.

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

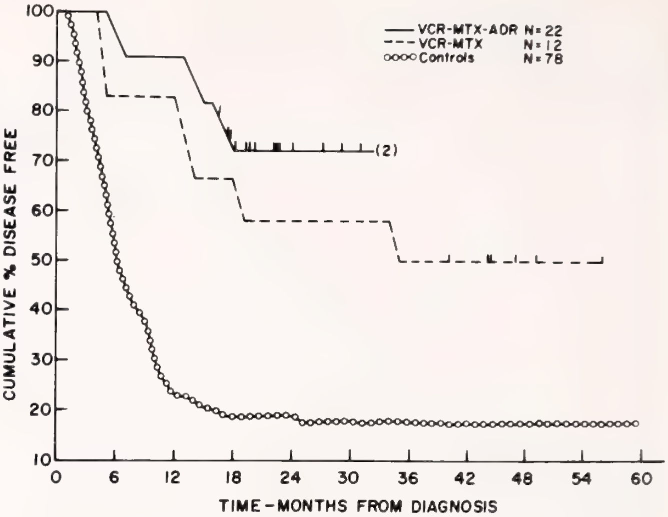
was given as a priming dose because of experimental evidence that this drug, at concentrations that are probably achieved in vivo, increases in vitro uptake of [^{3}H]MTX (*7*). The efficacy of vincristine in this setting has not been demonstrated in the clinical trial context. With increasing experience and compelling evidence that the high-dose MTX–CF program could be delivered without myelosuppression and with the evidence that adriamycin was effective in advanced OGS and when given alone as an adjuvant (*8*), our second protocol was developed. This protocol involved the use of both programs with minimal compromise in dose rate (third program in text-fig. 1) (*9*). In the hematologic neoplasms, major progress has been achieved with combinations of chemotherapeutic agents with which full doses could be delivered because of lack of additive dose-limiting toxicity (*10*).

The following represents a summary of the categories of progression of OGS. It applies in general to most categories of neoplastic disease. The disease progresses from the primary lesion to micrometastases and finally to overt metastatic disease. As above, the medical and pediatric oncologists are usually initially involved in the development of effective programs for overt metastatic disease. Once this is achieved, and if micrometastatic disease represents a significant problem, such treatment is applied as adjuvant chemotherapy shortly after control of the primary lesion. Since control of both primary lesion and micrometastatic disease is equally important, the term adjuvant is unfortunate and multimodality treatment is perhaps better. However, the latter is insufficiently precise and adjuvant chemotherapy remains the popular term.

Evolution of treatment of OGS

- Primary lesion
  - Past: Amputation, radiotherapy
  - Current approaches:
    1) Initial intensive chemotherapy designed to shrink primary lesion
    2) Subamputative surgery
- Micrometastases
  - Past: No treatment
  - Current: Systemic (adjuvant) chemotherapy, immunotherapy
- Overt metastatic disease
  - Past: Palliation
  - Current approaches:
    1) Systemic chemotherapy ± surgery ± X-ray treatment designed to create NED
    2) "Adjuvant" chemotherapy for microscopic disease

The results of our first 2 protocols of adjuvant chemotherapy of OGS are presented text-figure 2 (updated from original reports) (*9, 11*). In the historical control curve, the diseases of 80% of the patients have relapsed by 12 months and the cure rate (leveling off of the curve) is between 15 and 20%. In our experience and in the experience of six other institutions or groups who have recently carefully analyzed their data, no change has occurred in the historical control curve as determined from 2-year survival data over the years and up to the present time or to the time when adjuvant chemotherapy studies were initiated (*12*). One important exception to this experience exists wherein the control curve indicates a 45% survival in 2 years (Mayo Clinic: Unpublished data).

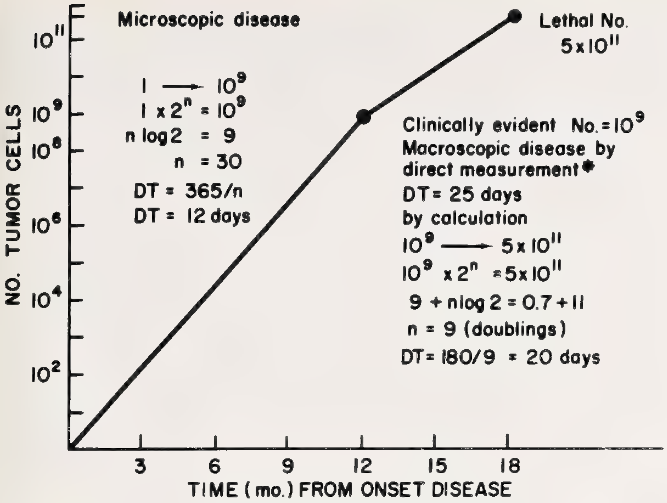


TEXT-FIGURE 2.—OGS. Disease-free survival. VCR=vincristine; ADR=adriamycin.

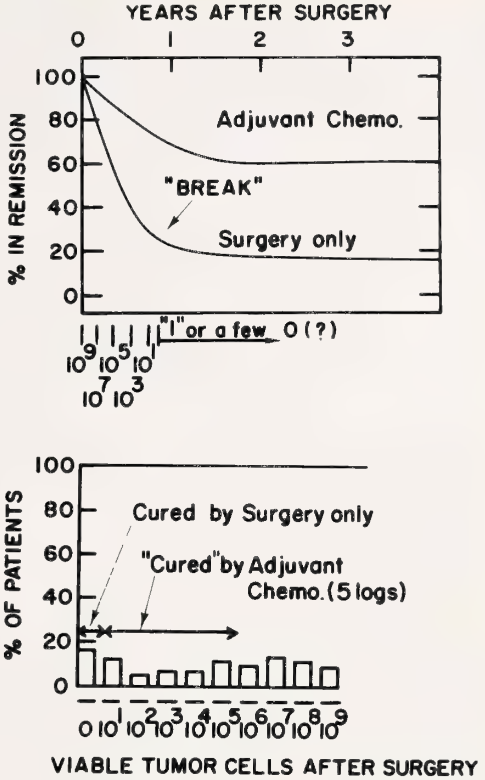
For the initial study, i.e., MTX–CF, the curve has plateaued at 50% and the minimum follow-up for that group of patients is 3 years (text-fig. 2). For the second study (MTX–CF plus adriamycin), the actuarial curve at 3 years is at 73% with a minimum follow-up of 14 months and a maximum follow-up of 33 months. Though the differences between the MTX–CF curves and the MTX–CF plus adriamycin curves are not yet statistically significant, they are consistent with an additive therapeutic effect. Similar results were achieved in five other studies of adjuvant chemotherapy in OGS. Thus the leveling off of the curve was between 45 and 60% and late relapses were infrequent (*8, 11, 13–16*). These results are consistent with the interpretation that the cure rate for OGS has been increased from 20 to 40–60% by adjuvant chemotherapy.

To achieve a better understanding of the dynamics of micrometastatic disease, we extrapolated and applied cytokinetic data from experimental adjuvant chemotherapy studies. These extrapolations derive largely from the experimental work of Skipper et al. (*17, 18*) and Lloyd et al. (*19*) at the Southern Research Institute.

Both by intuition and as a result of experimental adjuvant studies, one can logically suggest that the break in the untreated control curve in text-figure 2 represents the time required for one or a few clones of neoplastic cells to expand to a clinically recognizable number. This duration of OGS is 12 months. If one assumes that approximately 10^9 cells in the lungs would be roentgenographically evident, then the calculations made for microscopic disease in text-figure 3 are applicable. The number of doublings required to go from 1 to 10^9 cells is 30. If it takes 365 days to achieve this increase, then the tumor cell doubling time would be



TEXT-FIGURE 3.—OGS cytokinetics. *See* (*22*). DT=doubling time.



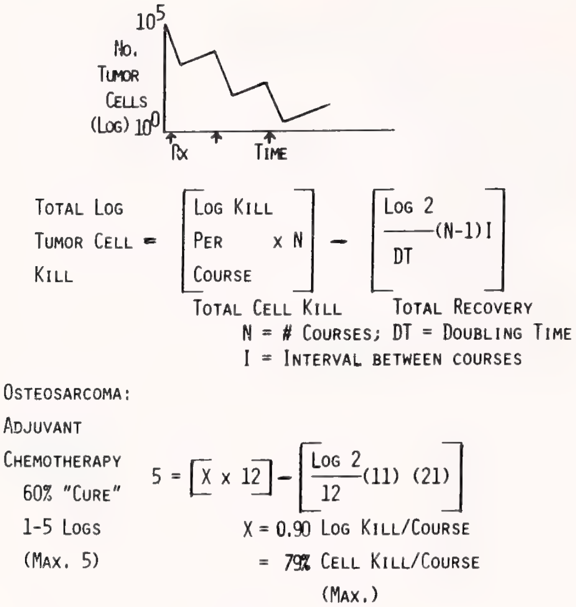
TEXT-FIGURE 4.—OGS. Determination of microscopic disease from remission curve.

12 days. Approximately 1×10^{12} cells represent the number present in patients with clinically evident acute leukemia (*20*). In OGS, metastatic disease is largely confined to the lungs and the lethal number is approximately 5×10^{11} (≈500 g of neoplastic cells). The median time from the development of roentgenographically evident pulmonary metastases to death is 6 months (*21*). By direct analysis of the doubling time of pulmonary metastases on serial radiograms, Band et al. (*22*) found the doubling time to be 25 days. From our calculations, a figure of 20 days is derived. These calculations (text-fig. 3) are consistent with the known Gompertzian dynamics of solid tumors; i.e., the doubling time of tumors increases with increasing tumor size (*17*).

A kinetic interpretation of the adjuvant OGS studies is presented in text-figure 4. This approach involves the extrapolation of an approach that has proved effective in in vivo adjuvant experimental models (*17–19*). In such systems, the number of persisting neoplastic cells after control of the primary lesion can be calculated, with reasonable accuracy and confirmed by bioassay, from the nature of the survival curve. Thus, as indicated above, the break point in the curve is the time beyond which surviving patients are presumed cured. Such patients, therefore, had no tumor cells capable of expanding indefinitely and producing relapse after control of the primary lesion. For surgery only, the disease-free survival curve levels off at 16%. Patients with greater than 10^9 cells would have had clinically evident or roentgenographically evident disease by chest film. Therefore, the distribution of patients in terms of number of neoplastic cells persisting after amputation varies between 0 and 10^9. By the analysis of the descending portion of the disease-free survival curve, a distribution of post-surgery cell load can be developed (text-fig. 4). With adjuvant chemotherapy, the plateau of the disease-free survival curve is at 60% as compared to 16%. To encompass tumor cytoeradication in 60 of the patients, we would have to cure those patients with up to 10^6 cells. If these calculations are correct, such chemotherapy would be capable of eradicating up through five logs of neoplastic cells (text-fig. 4).

When further knowledge of the biology of micrometastases becomes available, the above- and below-mentioned hypothetical considerations and extrapolations will prove to be an oversimplification. However, they do provide a means by which various factors that seem relevant to the construction and outcome of treatment may be examined. By the deliberate modification of these factors, the validity of a given hypothesis can be at least roughly tested by clinical studies.

An additional expression of the cytokinetic aspects of adjuvant chemotherapy of OGS is presented in text-figure 5. In the top portion of the text-figure, a schematic diagram of cytoreduction with intermittent chemotherapy is presented. The curve is composed of two major components: 1) cytoreduction achieved by the course of chemotherapy and 2) proliferative recovery in the interval between courses of treatment. The overall effect of an intermittent treatment program is a function of these two variables. The total log tumor cell kill is the product of the log kill per course and the number of courses, whereas the recovery between courses is inversely related to the doubling time and directly related to the number of courses and the interval between courses. From our previous analysis, a maximum of five logs of tumor is killed by adjuvant chemotherapy, and the doubling time of micrometa-



10^5
No. Tumor Cells (Log)
10^0
Rx
Time
Total Log Tumor Cell Kill = [Log Kill Per Course x N] − [Log 2 / DT (N-1) I]
Total Cell Kill
Total Recovery
N = # Courses; DT = Doubling Time
I = Interval between courses
Osteosarcoma: Adjuvant Chemotherapy 60% "Cure" 1-5 Logs (Max. 5)
5 = [X x 12] − [Log 2 / 12 (11) (21)]
X = 0.90 Log Kill/Course
= 79% Cell Kill/Course (Max.)

TEXT-FIGURE 5.—Adjuvant chemotherapy: Kinetics.

static OGS is 12 days. With these substitutions, the log tumor cell kill per course is 0.9 and the percent kill per course is 79. While this effect is greater than can be achieved in patients with overt disease, it is consistent with what might be expected in the kinetically more active and thus more sensitive micrometastatic disease.

Further analyses are presented below. Since total log tumor cell kill sufficient to produce cure is the goal, the variables that influence this are separately analyzed. Total log tumor cell kill varies directly with log cell kill per course. For experimental homogeneous populations of tumor cells, a linear-log relationship exists between dose and tumor cell kill (*20*). Thus a twofold increase in dose might double the log tumor cell kill. Clearly, better drugs such as improved adriamycin (*23*) might lead to an increased log tumor cell kill per course. Finally, combinations of agents, particularly those wherein the dose rate of agents individually is minimally compromised, would be expected to increase the log tumor cell kill per course. The preliminary results of the MTX–CF plus adriamycin regimen are consistent with this thesis (*see* above and text-fig. 2).

Adjuvant chemotherapy: Treatment variables

$$\text{Total log tumor cell kill} = \underbrace{[\text{Log kill per course} \times N]}_{\text{Total cell kill}} - \underbrace{\left[\frac{\text{Log } 2}{\text{DT}}(N-1)I\right]}_{\text{Total cell recovery}}$$

N=No. of courses; DT=doubling time; I=interval between courses

Total log tumor cell kill varies:

1) Directly with cell kill per course
   A) Dose
   B) Better drug(s)[a]
   C) Combinations[b]
2) Directly with No. of courses
3) Directly with doubling time
4) Inversely with interval[c]

[a] AD-32 (adriamycin analog).

[b] MTX–CF every 3 wk+adriamycin.

[c] MTX–CF every wk.

The total tumor cell kill would vary directly with the number of courses. As indicated below, however, it seems unlikely that one can exploit this with respect to long-term chemotherapy. As one might anticipate, the log tumor cell kill varies directly with the doubling time. Thus the shorter the doubling time, the greater the proliferative recovery in the interval between treatments. Thus the minimization of the interval between treatment courses by the use of weekly high-dose MTX–CF programs should result in a greater tumor cell kill. The superior effect of the weekly MTX–CF program in overt OGS (table 1) is consistent with this thesis (*6*). These considerations have, in fact, been employed in the generation of our second and third adjuvant chemotherapy protocols for OGS.

The above approach involves a number of assumptions, the importance of which will become increasingly apparent with the application and adaptation of a model to clinical trials and experimental data. One important variable involves drug resistance. It has been observed for a number of different tumor cells in culture that approximately 1 in 10^6 (10^{-6}) cells of a given population is resistant to a given drug (*24*). Compelling in vivo experimental evidence supports this (table 2). In this study of L1210 mouse leukemia cells treated with Ara-C, inoculum sizes of 10^5 cells can apparently be cured by the drug. However, inoculum sizes of 10^7 cells are not cured by the same treatment and the cells recovered after the end of treatment are resistant to subsequent exposure to Ara-C. Inoculum sizes of 10^6 are intermediate. These data are consistent with substantial in vitro data that 10^{-6} cells are drug-resistant and this population is selected out by the treatment of native populations of 10^6 or larger (*17–19*).

When these data of experimental studies of drug resistance are considered with respect to the data pre-

TABLE 2.—*L1210 leukemia: Drug resistance (typical results observed when L1210 leukemia is treated with Ara-C)*

No. of leukemia cells at initiation of treatment	No. of causes of Ara-C[a]	Resistance of leukemia cells to Ara-C after end of treatment[b]	Animals cured %
10^5	1	No	50
10^5	2	—	90–100
10^5	4	—	90–100
10^7	1	Yes	0
10^7	2	Yes	0
10^7	4	Yes	0
10^7	6–12[c]	Yes	0

[a] Every 3 hr (8 times), every 4 days.

[b] Demonstrated by passage of cells after treatment and retesting of response to Ara-C. Such cells are not cross-resistant to other classes of drugs.

[c] Good increases in host survival times but all animals died during maximum tolerated treatment. After 2–4 courses, the die is cast; cure has been achieved or failure is inevitable.

sented in text-figure 4, it is titillating to speculate that the 60% ceiling rate on disease-free survival is a function of the fact that 40% of patients have 10^6 or more tumor cells, which means that drug resistance in one or more cells would be obtained and cure would not be achievable with single-agent therapy.

The above calculations were obtained for drug-resistant cells in the initial unexposed population. Most antitumor agents are variably mutagenic so that, during treatment, mutant daughter cells are produced that have a higher potential for being genetically drug-resistant. Several in vitro studies indicate that such chemical mutants will increase the population of drug-resistant cells by a factor of 100. Thus, for daughter cells produced during treatment, 1 in 10,000 (10^{-4}) will be resistant (text-fig. 6). Treatment failure due to drug resistance may occur if the initial population of micrometastatic cells is greater than 10^6 or if the number of daughter cells produced during treatment is greater than 10^4.

ASSUME FRACTIONAL RESISTANCE = 10^{-6}

THEN:

CURE IMPOSSIBLE IF INITIAL CELL BURDEN $> 10^6$

ASSUME FRACTIONAL RESISTANCE OF DAUGHTER CELLS PRODUCED DURING TREATMENT = 10^{-4}

THEN:

CURE IMPOSSIBLE IF NO. OF DAUGHTER CELLS PRODUCED, AS CALCULATED FROM

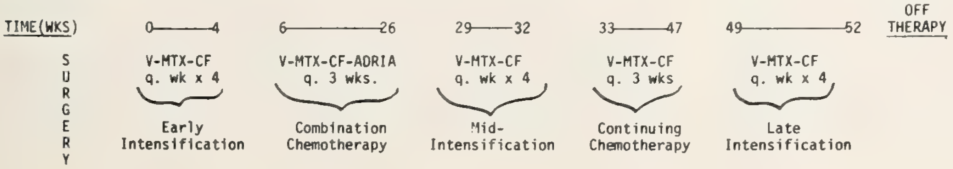
$$\left[\frac{\text{Log } 2}{\text{DT}} (\text{I})\ (\text{N-1})\right], \quad \text{IS} > 10^4$$

TEXT-FIGURE 6.—OGS cells. Drug resistance.

These latter considerations relating to the number of daughter cells produced during treatment provide a strong argument for intensive adjuvant treatment designed to maximally reduce and eliminate tumor cells as soon as possible. For example, if one starts with 10^5 cells in the adjuvant situation and each treatment course produces a 1-log reduction, nine courses will be required before cytoeradication is achieved in accordance with the formula presented in text-figure 5. During such treatment, 5×10^4 daughter cells would have been produced which, if drug resistance is factored into the equation, would mean treatment failure (text-fig. 6). However, if the treatment is modified (intensified) such that a 2-log reduction is achieved with each treatment course, only three courses of treatment are required for cytoeradication, and approximately 4,000 daughter cells would have been produced during treatment, a number that is below the theoretical threshold necessary for the selection of resistant daughter cells.

Our third chemotherapy adjuvant protocol for OGS started 1 year ago and is based on some of the above considerations (text-fig. 7). As was already indicated, weekly high-dose MTX–CF treatment is more effective in advanced disease than a triweekly program and presumably would effect a more rapid cytoreduction of micrometastatic disease and a decreased chance of the emergence of drug-resistant cells. Accordingly, an initial period of intensification occurs with weekly MTX–CF treatment. For practical reasons, such treatment cannot be extended beyond 4 weeks. After this, combination chemotherapy without the dose rate of either program being compromised is delivered in the form of adriamycin and high-dose MTX–CF triweekly, similar to that in protocol 1. Further cases and follow-up will be required to evaluate the effectiveness of this program as compared to protocols 1 and 2.

If micrometastases are eradicated by adjuvant chemotherapy in a substantial proportion of patients, it seems reasonable to assume that the number of such metastases is reduced in patients who eventually have recurrent disease. Our preliminary studies indicate that this is, in fact, so. Also, the time until the development of overt metastatic disease in patients receiving adjuvant chemotherapy as compared to the nonadjuvant control patients is delayed. This fact plus the increasing effectiveness of systemic treatment has led to a multidisciplinary curative intent treatment program for patients whose disease relapses (text-fig. 7) (*21*). The principle involves an initial combined chemotherapy and surgical attack on the pulmonary metastases designed to produce a state of NED. The chemotherapy cannot be specified, since it will depend in part on the chemotherapy employed in the previous adjuvant treatment. Multimodality treatment is initiated with chemotherapy or surgery depending on the clinical circumstances. Thoracotomy with resection of metastases is employed if chemotherapy fails to produce a complete response. Once NED is achieved, it is assumed that micrometastatic disease persists and adjuvant chemotherapy is continued for 18–24 months. In 21 patients thus treated, NED was achieved in 15 (table 3). The survival of such patients is presented in text-figure 8. The control curve is the time from the appearance of pulmonary metastases to death in patients seen prior to July 1972. For the patients given multimodality treatments, the curve is at 65% at 18 months and 12 of the 21 patients continue with NED. This curve is significantly superior to the control curve where the median survival is 6

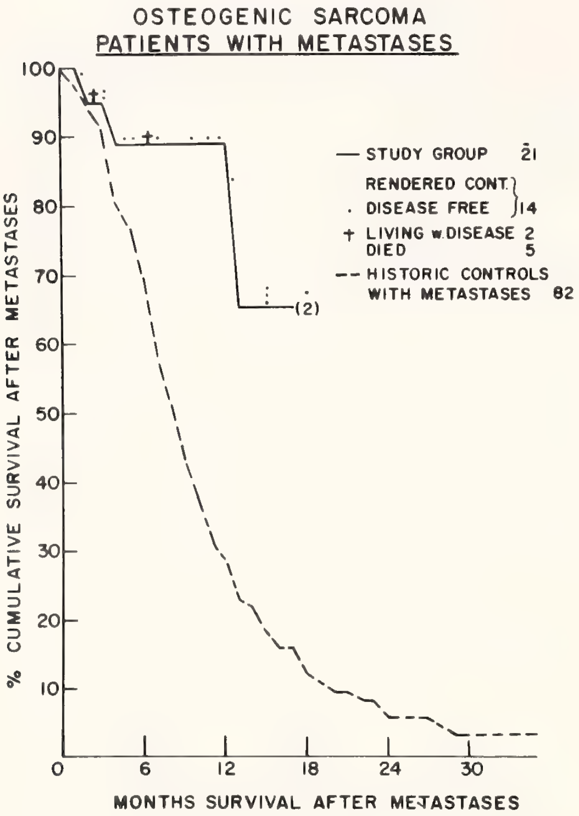


TEXT-FIGURE 7.—OGS. Adjuvant chemotherapy (CHMC-SFCI Study 3). V=vincristine; ADRIA=adriamycin.

TABLE 3.—*Overt metastatic OGS multidisciplinary treatment strategy, categories, and results*

Treatment sequence[a]	No. of patients	NED achieved in	NED continued
Surgery (chemotherapy 2 + XRT), chemotherapy 3	5	5	5
Chemotherapy 1, surgery, chemotherapy 3	3	3	2
Chemotherapy 1, surgery (chemotherapy 2 + XRT), chemotherapy 3	5	4	4
Chemotherapy 1, chemotherapy 3	8	3	3
Total	21	15	14

[a] One patient received adriamycin only. Chemotherapy 1: vincristine-MTX-CF every wk 4-8 times; chemotherapy 2: vincristine-MTX-CF every wk 4 times; XRT: X-ray treatment; chemotherapy 3: vincristine-MTX-CF every 2-3 wk for 18 mo. Two patients received concurrent adriamycin.



TEXT-FIGURE 8.—OGS survival from overt metastases.

months. These results are preliminary but promising (*21*).

The above calculations are based on the conclusions that the true cure rate today for surgery only in primary OGS is 20% and that, with the delivery of adjuvant chemotherapy, it is 40–60%. Although it is essential to develop and apply better treatment programs, it is also essential to be reasonably certain that previous programs, the programs upon which you hope to build, do in fact represent an advance. This problem is particularly difficult in adjuvant chemotherapy studies of OGS because 1) it takes a minimum of 2 and probably as much as 4 years to know with reasonable confidence whether this new program is superior to the previous one, and 2) even given this time frame, the number of cases—at least within a given institution—will be small so that significant differences, if they are to be detected, must be of considerable magnitude. Included among the problems of interpreting treatment progress in OGS are the historical control and histopathology.

In one study involving the analysis of cases reviewed through 1974, the 2-year disease-free survival progressively increased in the last several years to 40% (Mayo Clinic: Unpublished data). This observation is difficult to interpret. It is not consistent with careful studies performed in six other institutions wherein the prognosis, in the absence of adjuvant treatment, in terms of 2-year disease-free survival remains between 10 and 25% (*12*). It cannot be explained by the screening out of patients with negative conventional chest films but with positive tomograms. Such patients would clearly bias the sample in a favorable direction but they are extremely infrequent. For example, only one such case has occurred in the last 53 patients presenting with OGS at the Sidney Farber Cancer Institute. It should be emphasized that the other 6 institutions have been involved in adjuvant chemotherapy in recent years so that their analysis of nonadjuvant patient accrual goes up to 1972 or 1973. This fact has led to a reexamination of the need for a randomized nonadjuvant control group. This position is unacceptable to some, and probably most, investigators within institutions where 2-year disease-free survival rates have increased from 20% to 40–60% as a result (or at least concurrent with the use) of adjuvant chemotherapy.

Another major problem relates to histopathologic classification. As long as treatment for a given neoplastic disease remains static, the same is likely to be true of the histopathologic classification. Once treatment perturbations are introduced, histopathologic prognostic categories emerge or assume increasing importance. Since pathologic interpretation is difficult to objectify and quantify, it becomes even more important to attempt to achieve uniform criteria for histopathologic classification. Among other things, such classification is essential for the evaluation of a given treatment and comparison of such treatments to other treatment programs in different samples of patients in the same or different institutions. This evolution has occurred in almost all neoplastic diseases wherein progress in treatment is ongoing. Perhaps the most notable examples are the malignant lymphomas.

The pathologic material on the 12 patients in our initial adjuvant study has been rereviewed by a committee of three eminent bone pathologists (table 4). The 12 patients, the referring pathologist's diagnosis, the institutional pathologists' diagnoses, and the diagnosis of the committee and of additional consultants are listed. Patients 1–6 did not develop metastases and patients 7–12 did.

TABLE 4.—*OGS: Pathologic diagnosis*

Patient No.	Sex	Referring	Institutional	Committee	Consultant	Consultant	Comment	Metastases
1	F	—	OGS	Telangiectatic OGS	—	—	—	–
2	M	OGS	Sclerosing OGS	OGS	—	—	Committee review of postradiation specimen only[a]	–
3	M	Parosteal OGS	OGS, exophytic chondrosarcoma	Chondrosarcoma	—	—	OGS in medullary cavity of amputated section[a]	–
4	M	—	Biopsy–exophytic chondrosarcoma; osteosarcoma	Chondroblastic OGS	—	—	OGS confirmed in amputated section	–
5	M	OGS	OGS	Telangiectatic OGS	—	—	—	–
6	M	Parosteal OGS	Periosteal chondrosarcoma	Parosteal chondrosarcoma	Parosteal osteochondrosarcoma	Parosteal osteochondrosarcoma	Invasion into medullary cavity	–
7	F	Pleomorphic sarcoma	OGS	Osteoblastic OGS	Fibrosarcoma; spindle cell malignant tumor	Malignant fibrous histiocytoma	OGS in pulmonary metastases	+
8	M	OGS	OGS	—	—	—	—	+
9	M	—	OGS	Anaplastic OGS	—	—	—	+
10	F	OGS	OGS	—	—	—	—	+
11	F	—	OGS	Chondroblastic osteoblastic sarcoma	—	—	—	+
12	M	OGS	Telangiectatic OGS	Osteoblastic OGS	Benign bone cyst	Giant cell reparative granuloma	—	+

[a] Biopsy specimen differed substantially from amputated specimen.

There was good agreement on the diagnosis of classical OGS in patients 1, 2, 5, 8, 9, and 10. This includes 3 of 6 whose disease did not relapse and 3 of 6 whose disease did. A diagnosis of "highly vascular telangiectatic OGS" was made twice by the committee. This is the variant of OGS that has the poorest prognosis. The disease of neither of these patients relapsed. Patient 3 was considered to have chondrosarcoma by the committee, who, however, did not review the slides of the medullary cavity of the amputated section that showed OGS. This was also true of patient 4. Patient 6 was controversial. In this patient, the tumor developed as a parosteal lesion but progressive and finally extensive invasion into the medullary cavity occurred by highly undifferentiated tissue that demonstrated chondroid and osteoid elements.

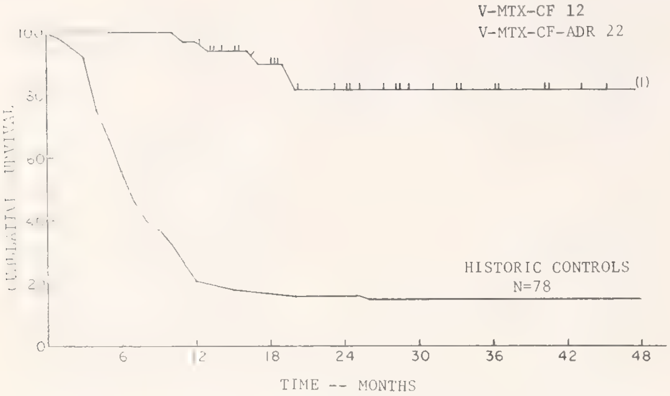
Patient 7 was thought to have other mesenchymal tumors by two consultants, but the histopathologic demonstration of OGS in the pulmonary metastases is presumably definitive.

Patient 11 was considered by the committee to have prominent cartilagenous as well as osteoblastic elements. Patient 12 was thought to have benign lesions by two consultants.

Clearly, in approximately half the patients in this sample, where the institutional diagnosis was considered classical OGS, some variation in terms of classification might be made by other distinguished bone pathologists. On careful reanalysis of the data presented in table 4, we contend that at least 5 of the 6 patients whose disease did not relapse had poor prognoses—OGS that could be considered "classic" or a slight variant thereof. All 6 patients whose disease did relapse would fall into that category.

In summary, we believe that the above supports the position tht the high-dose MTX–CF program, effective in advanced OGS, is effective in patients with microscopic disease. The number of patients included in the study was small; in retrospect, it would have been better to have continued case accrual. Most certainly, the above analysis indicates the need for a continuing prospective review of pathologic material in adjuvant studies. Ample precedent for this exists in lymphoma studies. Such a process will progressively define and refine the classification of variants of OGS and their prognostic import.

The final arbiter of the effect of a new treatment program on a category of cancer is survival. We have discussed objective response rate, complete response rates, and tumor-free survival. The overall survival of patients with OGS treated before and after July 1972 (the time we initiated our adjuvant studies) at the Sidney Farber Cancer Institute–Children's Hospital Medical Center is presented in text-figure 9. The lower curve includes the historical control patients and levels off at 16%. For the 34 patients treated on our first two adjuvant protocols (the third protocol was introduced only 1 yr ago—too short to impact on survival), the survival curve levels off at 24 months and remains at 80% for 48 months. OGS is a rare disease and even at a



TEXT-FIGURE 9.—OGS. Effective adjuvant chemotherapy on survival. V=vincristine; ADR=adriamycin.

large center case accrual is such that it is difficult to ask a quantitative question. For example, although MTX-CF is clearly highly effective in advanced OGS, only 12 patients were included in the adjuvant study. In the second study, adriamycin was added to MTX-CF. This study taken with the first supports the thesis that adjuvant chemotherapy improved survival as compared to historical control patients but the question as to whether the combination is superior to MTX-CF only remains open because of insufficient follow-up (*see* above). Finally, the third study which may have an impact on long-term survival is the multimodality treatment program for patients whose disease relapses. This study is positive (text-fig. 6) but preliminary. In short, even though the results of these three independently analyzed programs present problems in interpretation, primarily because of small case numbers and/or insufficient follow-up, the overall impact on survival is highly significant and substantial (text-fig. 9).

Major progress has been achieved in the palliative and definitive treatment of patients with OGS. As for all categories of neoplastic disease in the early stages of therapeutic advances, the interpretation of studies presents problems and the disease is increasingly recognized as being more heterogenous clinically, pathologically, and prognostically.

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ADDENDUM[1]

In the above "Guest Editorial," we reported the results of adjuvant chemotherapy studies of OGS performed at the Sidney Farber Cancer Institute–Children's Hospital Medical Center. The following is an updated summary of that experience.

The three adjuvant studies used for OGS are presented in text-figure 1. The updated results of these three studies on disease-free survival are depicted in text-figure 2. When they presented to the Sidney Farber Cancer Institute–Children's Hospital Medical Center, patients with OGS in whom the primary tumor could be controlled were accrued to study #1, 4 to 6 years ago; study #2, 2 to 4 years ago; and study #3, the past 2 years. Thus the maximum follow-up is approximately 6, 4, and 2 years, respectively.

Our historical control data involve 78 consecutive patients accrued prior to July 1972. Historical controls for OGS were discussed at length in the Editorial. In summary, we have not found that disease-free survival in our historical control patients improved up to 1972, and scientists at other institutions who analyzed their data, with one exception (*1*), also found that the plateau for disease-free survival is approximately 20% and that no change or improvement in disease-free survival has occurred for the past 20 years prior to the use of adjuvant chemotherapy (*2–5*). Moreover, the usual factors which complicate the use of historical controls, such as imprecise parameter of response, changing criteria for the selection of patients, and changing schema for the selection and evaluation of patients, that apply to certain other categories of neoplastic disease apply minimally, if at all, to OGS (*see* Editorial).

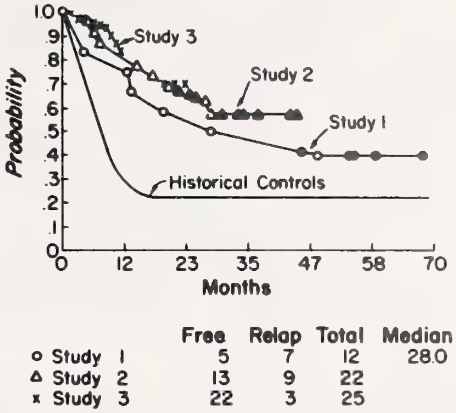
Study #1

This study, which involves MTX–CF only (text-fig. 1), comprises 12 consecutive patients; the disease has relapsed in 7, in 1 as late as 4 years after control of the primary. The median time to relapse was 34 months as compared with 6 months for the historical controls. Statistical analysis (double Wilcoxon) indicates that disease-free survival is significantly improved for this study compared with the historical controls ($P < 0.05$).

These results of study #1 have been confirmed. In a study at the National Cancer Institute, the disease-free survival curve for high-dose MTX–CF patients leveled off at 40 to 45% and was significantly superior to concurrent and historical (albeit nonrandomized) controls (Rosenberg S: Unpublished observations).

Study #2

Because adriamycin was demonstrated to be effective in the adjuvant setting for OGS (*6*) and because with proper attention to pharmacology and supportive care MTX–CF could be given without myelosuppression (*7*), we combined adriamycin at full doses with MTX–CF with minimal compromise in dose rate. Combinations of agents with qualitatively different dose-limiting toxicity such that full doses can be delivered have been an important and successful basis for combination chemotherapy in other categories of neoplastic disease, particularly the hematologic types of cancer (*8*). This curve is everywhere superior to the study #1 disease-free survival though the difference is only questionably significant ($P > 0.05$ and < 0.1).



	Free	Relap	Total	Median
o Study 1	5	7	12	28.0
△ Study 2	13	9	22	
× Study 3	22	3	25	

TEXT-FIGURE 1.—OGS: Disease-free survival with adjuvant chemotherapy (time from control of primary relapse). *Open symbol* = relapse; *closed symbol* = at risk.

Study #3

In patients with advanced metastatic OGS, weekly MTX–CF produced a response rate of approximately 70% as compared with 35% for triweekly MTX–CF (*9*). Intuitively and experimentally, that treatment which is most effective against advanced neoplasia should be most effective in the adjuvant setting. In view of this hypothesis and of the evidence supporting early and late intensification for treatment of micrometastases, study #3 included 4 weekly courses of high-dose MTX–CF at the beginning, middle, and end of adjuvant chemotherapy, which was otherwise similar to that used for study #2 (*see* text-fig. 1). Twenty-five patients have accrued to that study over the past 2 years and in 3 the disease has relapsed. It is too early to evaluate disease-free survival for the patients in this study. The curve to date is comparable to that for study #2.

At 24 months, the actuarial disease-free survival curve for the control subjects is 18%, and 54, 64, and 70% for the patients in studies #1, 2, and 3, respectively.

In text-figure 2, disease-free and overall survival are compared for the 59 patients included in the studies. Starting 3 years ago and based in part on the observation that patients relapsing after adjuvant chemotherapy had a longer time until relapse and a smaller number of pulmonary metastases compared with historical controls, a multimodality program was initiated for patients relapsing after adjuvant chemotherapy. The principle of this program involved the creation of NED by chemotherapy, thoracotomy, and, in some instances, radiotherapy. Following creation of NED, chemotherapy designed to eradicate micrometastases was administered

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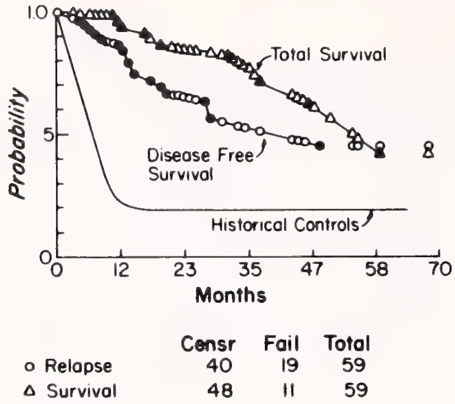
[1] The co-authors of this Addendum are Norman Jaffe, Hugh Watts, and Herbert Abelson.



Text-figure 2.—OGS: Disease-free and overall survival with adjuvant chemotherapy. *Closed sympol* = relapse or death (failure); *open symbol* = at risk.

for 6 to 12 months. The chemotherapy in this combined modality program had to be individualized and depended primarily on the prior chemotherapy the patients received. The curves in text-figure 2 indicate some deviation of the overall survival curve away from the continuous disease-free survival curve through 36 months, which is the duration of the combined modality study. These studies, as well as others, indicate that combined modality treatment for patients whose cancer relapses will improve survival, but whether anything more than that has been accomplished requires further follow-up. By life-table analysis of our historical control patients, 30% are surviving at 12 months and 23% at 24 months, after which the curve levels off at 20%. The comparable figures for 12 and 24 months for overall survival in text-figure 2 are 93 and 88%, respectively.

We conclude that major progress has been achieved in the treatment of OGS. As for all categories of neoplastic disease in the early stages of therapeutic advances, the interpretation of studies presents problems, and the disease is increasingly recognized as being more heterogeneous clinically, pathologically, and prognostically.

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GUEST EDITORIAL

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JNCI 52:3–7, 1974

Impact of Cancer: Years of Life Lost Due to Cancer Mortality

CANCER has risen from the eighth most important cause of death in the United States in 1900 to the number 2 cause of death in 1972, second only to diseases of the heart. The American Cancer Society estimates that 345,000 persons died of cancer in the United States in 1972. In evaluating this, we need to consider several aspects. First, it must be remembered that cancer is really many diseases, all related by their neoplastic characteristics but differing in their sites of origin, their cellular morphology, and their causes.

Incidence and prevalence data must be considered when the magnitude of the cancer problem is estimated in terms of prevention, control, and treatment efforts. Mortality and disability data must be considered in evaluation of the loss to society and the population, but measures should be sought that would make some allowance for the intuitive idea that death at age 75, for instance, does not represent as great a loss to society as death at age 35. The loss of life from cancer should not be considered as a total loss but only as a relative loss in relation to our life expectancy. An example of this type of consideration is the now standard calculation of relative survival rates rather than observed survival rates for cancer patients after therapy. The observed survival rates for these patients are compared with the expected survival probabilities for a group of people in the general population similar to the patient group with respect to sex, age, and calendar period of observation. The relative loss of lifetime, or life shortening, is often used as a criterion for evaluation of the long-term or late effects of hazardous agents such as ionizing radiation (*1*).

Cancer mortality data for the United States during 1950–1967 have been compiled and analyzed by Burbank in (*2*). In this reference book, age-specific death rates are plotted by sex and race for each cancer site. Typically, the curves show mortality rates increasing in the latter years of life. While the final objective of the National Cancer Program may be to completely eliminate cancer as a cause of death, it would be impractical and unreal for us to attempt to eliminate mortality from all other causes for cancer patients. Our immediate and attainable objective should be to prevent or delay cancer mortality and increase the life expectancy. In other words, our goal should be to decrease the life shortening caused by cancer. As we progress toward this goal, we will gradually shift the age-specific death rate curves to the right. In working toward this objective, we should evaluate screening programs and early diagnosis and treatment efforts on the basis of whether they really decrease the age-specific mortality rates, especially at younger ages, and increase the life expectancy for cancer patients, or whether they merely produce an apparent increase in survival time after early diagnosis.

In considering the possibility that all cancers could be completely eliminated, Dublin et al. calculated the resulting gain in expectation of life for each 10-year age group (*3*). The average length of life of white males, according to conditions in 1939–41, would be increased by 1.39 years, and that for white females by 2.05 years. The corresponding extensions of life expectancy for blacks would be 0.71 years for males and 1.40 years for females. Life expectancies have changed slightly since 1941, but not enough to significantly alter the value of these numbers and therefore they have not been recalculated here. Our main objective is to show how many years of life in the United States can be saved by controlling mortality from cancer.

Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

METHODS AND RESULTS

To evaluate the loss due to cancer mortality, the latest United States mortality data (1968) from the National Center for Health Statistics were combined with life expectancy data for the same population to calculate the person-years lost (*4*). The number of cancer deaths in each 5-year age group were multiplied by the average number of years of life remaining from the midpoint of the age interval.

Assuming the usual most productive period of a person's life is between the ages of 20 and 65, work-years lost during this age interval were computed. Since the life expectancy for every age group is greater than 65 years, people in each age group can be expected to complete a full working life; therefore any death before age 65 is considered a loss for the rest of that person's working life.

The numbers of person-years lost and work-years lost due to mortality from all malignant neoplasms in the United States in 1968 are shown by age group in tables 1 and 2. The average remaining lifetime values are used in computing the person-years lost, but they do not affect the work-years lost. The person-years lost for females are somewhat higher than for males despite more cancer deaths among men. This is a reflection of deaths among women at somewhat younger ages and the longer life expectancy among women than among men for any given age. The total work-years lost for males and females are essentially equal for this calendar period. The loss of life due to cancer for the most frequently affected sites and organ systems is shown in tables 3 and 4 for males and females, respectively.

TABLE 1.—*Male person-years and work-years lost from all malignant neoplasms in 1968 by age group*

Age group	Average remaining lifetime	Average working years* remaining	Deaths†	Person-years lost	Work-years lost
0–4	66.4	45.0	733	48,671	32,985
5–9	61.6	45.0	823	50,696	37,035
10–14	56.7	45.0	619	35,097	27,855
15–19	52.0	45.0	842	43,784	37,890
20–24	47.4	42.5	795	37,683	33,788
25–29	42.9	37.5	878	37,666	32,925
30–34	38.3	32.5	1,131	43,317	36,758
35–39	33.8	27.5	2,051	69,324	56,403
40–44	29.4	22.5	4,110	120,834	92,475
45–49	25.2	17.5	7,573	190,840	132,528
50–54	21.2	12.5	12,548	266,018	156,850
55–59	17.7	7.5	18,633	329,804	139,748
60–64	14.5	2.5	24,122	349,769	60,305
65–69	11.7	0	26,639	311,676	0
70–74	9.4	0	26,923	253,076	0
75–79	7.4	0	22,489	166,419	0
80–84	5.7	0	14,237	81,151	0
85+	4.7	0	8,519	40,039	0
Totals			173,665	2,475,864	877,545

*Based on working years between 20 and 65 years remaining after midpoint of each age group.

†29 deaths were excluded because age was not stated.

TABLE 2.—*Female person-years and work-years lost from all malignant neoplasms in 1968 by age group*

Age group	Average remaining lifetime	Average working years* remaining	Deaths†	Person-years lost	Work-years lost
0–4	73.5	45.0	602	44,247	27,090
5–9	68.7	45.0	645	44,312	29,025
10–14	63.8	45.0	502	32,028	22,590
15–19	58.9	45.0	510	30,039	22,950
20–24	54.1	42.5	584	31,594	24,820
25–29	49.3	37.5	728	35,890	27,300
30–34	44.5	32.5	1,359	60,476	44,167
35–39	39.8	27.5	2,818	112,156	77,495
40–44	35.2	22.5	5,417	190,678	121,883
45–49	30.8	17.5	9,059	279,017	158,533
50–54	26.5	12.5	12,413	328,945	155,163
55–59	22.4	7.5	15,107	338,397	113,303
60–64	18.5	2.5	16,930	313,205	42,325
65–69	14.9	0	18,448	274,875	0
70–74	11.6	0	19,230	223,068	0
75–79	8.7	0	18,008	156,670	0
80–84	6.2	0	12,681	78,622	0
85+	4.9	0	9,789	47,966	0
Totals			144,830	2,622,185	866,644

*Based on working years between 20 and 65 years remaining after midpoint of each age group.

†23 deaths were excluded because age was not stated.

TABLE 3.—*Male deaths, person-years lost, and work-years lost from cancer in 1968* (*by site*)

Site	Deaths	Person-years lost	Work-years lost*
All sites	*173, 665*	*2, 475, 864*	*877, 545*
Buccal cavity and pharynx	*5, 382*	*79, 101*	*28, 815*
Tongue	1, 250	17, 699	6, 038
Pharynx	2, 466	38, 136	14, 540
Digestive organs	*50, 320*	*631, 996*	*176, 253*
Esophagus	4, 360	60, 853	19, 275
Stomach	10, 330	123, 747	31, 765
Small intestine	341	5, 051	1, 908
Colon	15, 689	190, 054	50, 303
Rectum	5, 841	71, 480	18, 798
Liver	1, 466	22, 005	8, 135
Gallbladder	1, 613	19, 157	4, 733
Pancreas	9, 914	128, 168	36, 830
Respiratory system	*52, 168*	*743, 823*	*239, 728*
Larynx	2, 532	35, 255	10, 838
Lung	48, 822	695, 083	222, 948
Breast	265	3, 683	1, 353
Genital organs	*17, 805*	*178, 272*	*33, 195*
Prostate	16, 843	151, 024	13, 015
Urinary organs	*9, 961*	*125, 726*	*35, 263*
Bladder	5, 983	63, 989	11, 540
Kidney	3, 978	61, 738	23, 723
Other sites	*19, 679*	*352, 287*	*169, 812*
Bone	1, 015	24, 333	15, 260
Skin	2, 621	44, 240	22, 078
Brain	4, 361	111, 124	67, 015
Thyroid	387	5, 747	2, 258
Leukemia	8, 173	178, 362	99, 958
Lymphoma	9, 912	182, 614	93, 168

*Based on life expectancy coverage for working years between ages of 20 and 65 years.

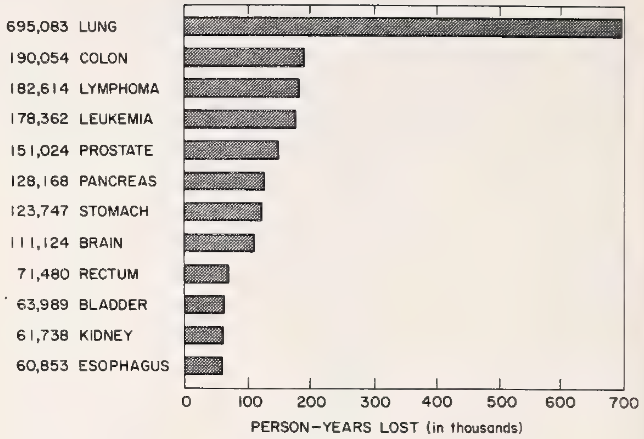
TABLE 4.—*Female deaths, person-years lost, and work-years lost from cancer in 1968* (*by site*)

Site	Deaths	Person-years lost	Work-years lost*
All sites	*144, 830*	*2, 622, 185*	*866, 644*
Buccal cavity and pharynx	*1, 911*	*33, 831*	*10, 735*
Tongue	453	7, 833	2, 275
Pharynx	700	14, 168	5, 030
Digestive organs	*43, 231*	*620, 830*	*134, 150*
Esophagus	1, 444	23, 506	6, 183
Stomach	6, 570	90, 778	19, 655
Small intestine	324	5, 127	1, 360
Colon	18, 337	261, 703	56, 660
Rectum	4, 564	66, 789	15, 293
Liver	730	13, 381	4, 425
Gallbladder	2, 996	39, 773	6, 120
Pancreas	7, 460	107, 355	21, 178
Respiratory system	*11, 305*	*211, 464*	*68, 805*
Larynx	303	5, 603	1, 810
Lung	10, 534	197, 047	63, 763
Breast	28, 810	569, 167	206, 975
Genital organs	*23, 122*	*448, 840*	*157, 963*
Cervix uteri	7, 106	158, 894	67, 690
Corpus uteri	5, 650	90, 033	22, 025
Ovary	9, 488	187, 197	65, 125
Urinary organs	*4, 827*	*73, 140*	*18, 385*
Bladder	2, 506	30, 239	4, 455
Kidney	2, 321	42, 900	13, 930
Other sites	*17, 563*	*355, 054*	*137, 168*
Bone	753	20, 507	10, 688
Skin	1, 779	35, 894	15, 178
Brain	3, 146	97, 513	50, 398
Thyroid	630	9, 412	2, 260
Leukemia	6, 199	156, 882	76, 015
Lymphoma	7, 862	152, 977	56, 448

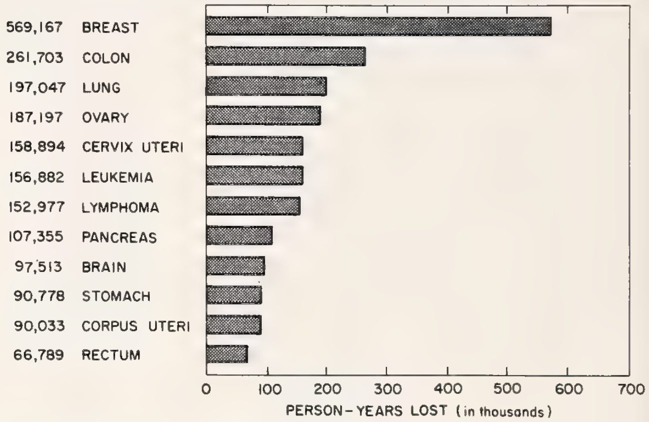
*Based on life expectancy coverage for working years between ages of 20 and 65 years.

A clearer portrayal of the relative importance of deaths from cancer of the major sites can be seen in table 5 and text-figures 1 and 2. For males, lung cancer is commonly recognized as the leading cause of cancer mortality, but it is not often realized that lung cancer results in more than 3 times as much lifetime loss as any other type of cancer (text-fig. 1). For males, the cancers causing the most serious loss of lifetime, after lung, are colon, lymphoma, leukemia, prostate, pancreas, stomach, and brain, in that order. A comparison of text-figure 1 and table 5 shows shifts in the order of importance of deaths and person-years lost among the major cancer types. For example, lymphoma ranked as the sixth leading cause of death, but is the third leading cause of person-years lost, while prostate dropped from the second leading cause of death to fourth place in person-years lost. Text-figure 2 shows that, in women, breast cancer accounts for more than twice as much loss of lifetime as any other type of cancer and colon cancer ranks second, as it does in men.

Deaths at older ages have relatively little effect on the loss of lifetime, whereas deaths at young ages have a great effect, with the resulting loss representing all of the person's remaining expected lifetime. Examples of the relative magnitudes of the person-years lost as compared to the number of persons dying in each age group are illustrated in text-figures 3 and 4 for cancer of the prostate and leukemia. A relatively small number of deaths early in life due to leukemia results in a large loss in terms of person-years over the expected lifetime. Most prostate cancer deaths occur after age 65, but the number of deaths from this cancer are of such mangitude that over 30,000 person-years are lost annually from the 70–74-year age group alone. The areas under the curves represent



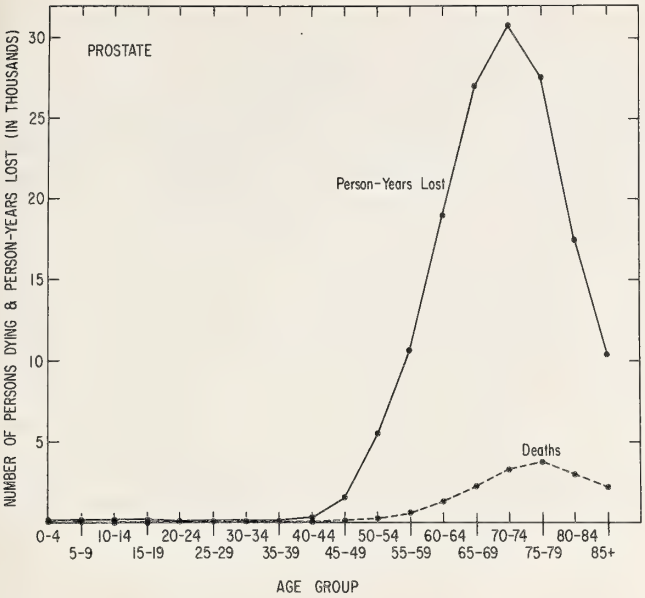
Text-figure 1.—Person-years lost from the leading types o cancer, 1968: males.



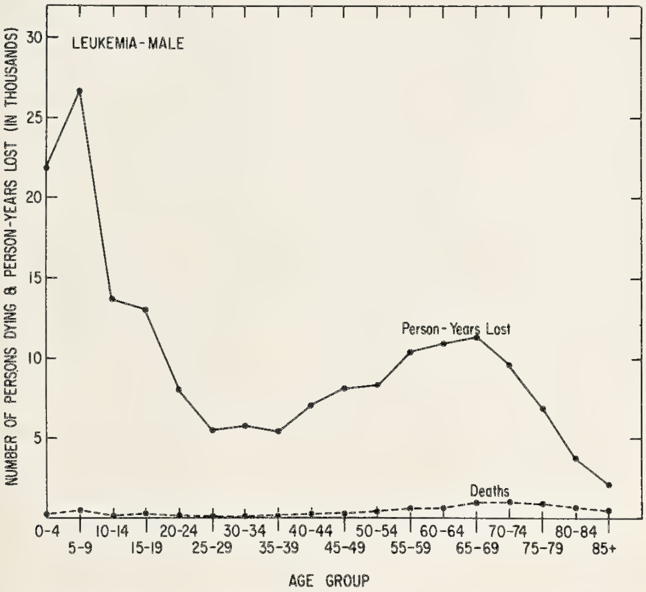
Text-figure 2.—Person-years lost from the leading types of cancer, 1968: females.

Table 5.—*Ranking of deaths from cancer in 1968 by site and sex*

Male			Female		
Rank	Site	Deaths	Rank	Site	Deaths
1	Lung	48, 822	1	Breast	28, 810
2	Prostate	16, 843	2	Colon	18, 337
3	Colon	15, 689	3	Lung	10, 534
4	Stomach	10, 330	4	Ovary	9, 488
5	Pancreas	9, 914	5	Lymphoma	7, 862
6	Lymphoma	9, 912	6	Pancreas	7, 460
7	Leukemia	8, 173	7	Cervix uteri	7, 106
8	Bladder	5, 983	8	Stomach	6, 570
9	Rectum	5, 841	9	Leukemia	6, 199
10	Brain	4, 361	10	Corpus uteri	5, 650
11	Esophagus	4, 360	11	Rectum	4, 564
12	Kidney	3, 978	12	Brain	3, 146
13	Skin	2, 621	13	Gallbladder	2, 996
14	Larynx	2, 532	14	Bladder	2, 506
15	Pharynx	2, 466	15	Kidney	2, 321
16	Gallbladder	1, 613	16	Skin	1, 779
17	Liver	1, 466	17	Esophagus	1, 444
18	Tongue	1, 250	18	Bone	753
19	Bone	1, 015	19	Liver	730
20	Thyroid	387	20	Pharynx	700
21	Small intestine	341	21	Thyroid	630
22	Breast	265	22	Tongue	453
			23	Small intestine	324
			24	Larynx	303



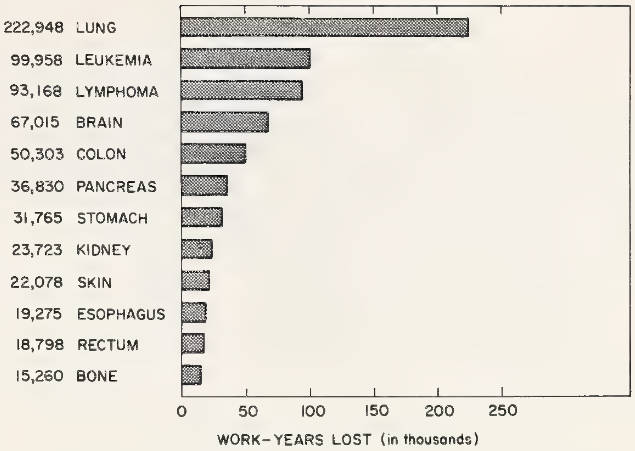
Text-figure 3.—Number of male deaths and number of person-years lost by 5-year age group from cancer of the prostate, 1968.



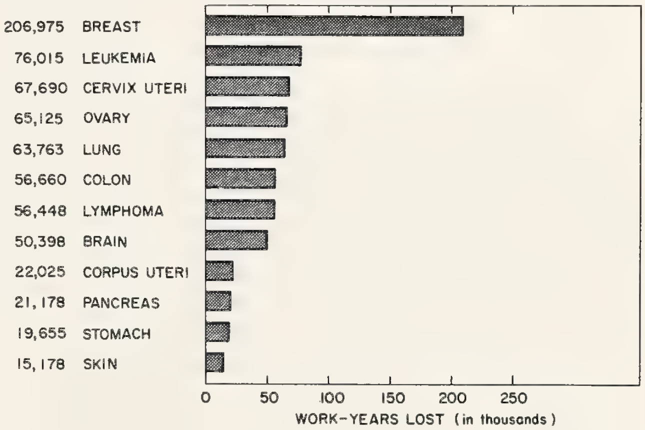
Text-figure 4.—Number of male deaths and number of person-years lost by 5-year age group from leukemia, 1968.

the total magnitude of these 2 cancer problems as evaluated, and points on the curves represent the loss relative to the age at death.

When the relative importance of work-years lost (text-figs. 5, 6) is compared to the number of deaths, deaths at earlier ages play an even more important role. Leukemia, for example, which accounts for one-sixth as many deaths as lung cancer in men, accounts for almost half as many work-years lost as lung cancer and is the second ranking cause of work-years lost for both men and women. Prostate cancer, on the other hand, which is the second leading cause of deaths, is not even within the first 12 leading causes of loss of work-years.



Text-figure 5.—Work-years lost from the leading types of cancer, 1968: males.



Text-figure 6.—Work-years lost from the leading types of cancer, 1968: females.

CONCLUSION

These examples indicate the magnitude of the cancer problem, which varies with each type of cancer; i.e., each type of cancer is a special problem. There are efforts to improve diagnostic and treatment methods but, while some progress is being made in these areas, the greatest potential for cancer control lies in the possibility of reducing incidence for all age groups through preventive measures. The costs to society in terms of person-years and work-years lost, as described here, are comparative measures which may be useful in the determination of priorities for research on various types of cancer.

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ADDENDUM

Our Editorial on the impact of cancer in terms of years of life lost due to cancer mortality in the United States during 1968 has been useful in the evaluation of the magnitude of the total cancer problem and the relative contribution from each cancer site. Because of the continuous increase in cancer mortality, we have updated our calculations of person-years lost based on the published data for 1972. The total person-years lost for females in 1972 were still higher than for males, despite more cancer deaths among males. For males and females, the sites at which cancer caused higher mortality included all those associated with smoking tobacco. Since 1968, deaths of women with lung cancer increased 42%, and the number of person-years lost increased 48%. In 1972, lung cancer in males resulted in almost four times as much lifetime loss as any other type of cancer. Although breast cancer still causes more than twice as many person-years lost as any other cancer in females, by 1972 lung cancer surpassed that of colon as the second leading cause of death. This emphasizes the growing importance of lung cancer in women that can be directly related to their smoking habits. The total cost of cancer in the United States in 1972 was estimated to be over $17 billion, and the financial losses from morbidity and mortality are three times as great as the direct medical expenses.

GUEST EDITORIAL

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Do Human Tumors Show a Chromosome Pattern Specific for Each Etiologic Agent?[3]

IN 1912, Boveri (*1*) proposed that chromosome changes are important in the etiology of tumors. As improved cytologic techniques were later developed, it became apparent that tumor cells have mitotic abnormalities such as nondisjunction and lagging chromosomes. For most tumors karyotypic changes vary among different individuals. Within a single individual, however, the chromosome pattern remains constant, though clonal evolution of abnormal karyotypes may occur. This constant pattern in an individual has led to a theory of tumor progression that the basic line or stemline in a tumor is clonal in origin and may contain a particular constant chromosome abnormality (*2*). Cells with this abnormality appear to have a selective advantage and proliferate, often at the expense of the normal cells. The use of genetic markers supports this theory of clonal origin for some tumors (*3*). In most instances, the chromosome changes denote *somatic mutations* in the affected tissues, since a normal karyotype is observed in unaffected cells.

The karyotypic variability suggested that the chromosome changes were merely an epiphenomenon and were not involved in the initiation of disease (*4*). Consistent abnormalities were found in two conditions, however. The Philadelphia (Ph^1) chromosome (previously thought to be a partial deletion of the long arm of one G chromosome) is found in the marrow cells of most patients with chronic myelogenous leukemia (CML) (*5*). One G chromosome is missing in tumor cells of most meningiomas (*6–8*).

New staining techniques with quinacrine fluorescence (*9*), Giemsa banding (*10*), or both, are useful for re-examining the karyotypes of affected cells. It is now possible to determine whether there is a nonrandom association between specific chromosome abnormalities and particular tumors or potentially malignant disorders. Furthermore, chromosome abnormalities regularly associated with a disorder can be distinguished from those that are sporadic.

I present a hypothesis proposing a specific relationship between chromosomes and etiologic agents of various tumors. The scope of the terms "tumor" and "etiologic agent" should be clarified. Whereas most specific pathologic processes discussed in this paper are tumors, the neoplastic nature of some is debatable; therefore, the term "disease" is used in some instances. For the same reason, the term "oncogenic" agent is too restrictive; the terms "mutagenic" or "etiologic" agent are more suitable.

The following postulates are based largely on data obtained from animal experiments. As will become evident later, it is impossible to perform many of these studies in man. However, data now available suggest that chromosome abnormalities in many diseases of man are not random.

POSTULATES

1) Susceptibility of an individual to a mutagenic agent is a function of his genetic constitution.

2) For individuals of a given species, each class of mutagenic agents produces a different pattern of chromosome abnormalities within cells from susceptible tissues.

3) The diseases produced in individuals of the same species by different classes of agents may appear clinically and histologically indistinguishable.

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Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

HYPOTHESIS

a) The chromosome pattern within affected cells may be consistent for a disease produced by a single etiologic agent (or possibly several closely related agents).

b) The chromosome pattern may be highly variable for a given disease that can be produced by different etiologic agents.

c) Within a disease category, it may be possible to distinguish individuals whose affected cells have the same chromosome abnormality and thus to identify those whose disease may be due to the same etiologic agent.

d) The converse of *c*)—that a single etiologic agent may cause the same chromosome abnormality in cells from each individual—is probably less valid for a genetically heterogeneous population.

Evidence suggesting a specific chromosome abnormality-"tumor" relationship for certain conditions in man is summarized in table 1. The affected cells have a relatively consistent chromosome pattern in CML, meningioma, and Burkitt's lymphoma. The chromosomes analyzed by standard techniques show a variable pattern in the affected cells of the other 3 conditions. Some of these observations have limited value because the techniques could not precisely identify the specific chromosomes involved and the sites of chromosome breakage and rearrangement. However, certain consistent "chromosome subgroups" may be detectable when cells from patients with the latter diseases are analyzed with more sophisticated cytogenetic techniques.

DISEASES WITH A CONSISTENT CHROMOSOME PATTERN

CML

The chromosome changes of peripheral blood and bone marrow leukocytes in patients with CML have been studied extensively with standard cytogenetic techniques, and a consistent pattern has emerged. Approximately 90% of these patients have the Ph^1 chromosome (*11, 12*); most of the remaining 10% have an apparently normal karyotype. These Ph^1-negative patients are clinically different: They are older, have lower leukocyte and platelet counts, and usually do not respond as well to busulfan treatment as do the Ph^1-positive patients (*11, 12*). Their life expectancy is shorter—15 months as compared with 40 months for Ph^1-positive patients (*11*).

Ph^1 is a chromosome #22 that is lacking most of its long arm (22q−) (*13, 14*); Rowley (*15*) recently observed that this material from 22q may be translocated to chromosome #9. One chromosome #9, with an additional band of dully fluorescent material at the end of its long arm (9q+), was found in all 23 patients with Ph^1-positive CML whose cells were examined with quinacrine fluorescence (*15, 16*). Additional chromosome abnormalities are superimposed on the Ph^1-positive cell line when the patient enters the terminal acute phase. The new techniques reveal that the chromosomes involved in the 3 most common karyotypic changes are nonrandom. The extra C chromosome in the 5 patients studied most closely resembled #8 (*15, 16*). The metacentric marker chromosome in all 8 patients contained at least 1 long arm of #17 (*15, 17*), and in 5 patients it was thought to be an isochromosome for the long arm of #17 (*17*). In 4 of 5 patients with the double Ph^1, the 2 Ph^1 chromosomes appeared identical (*15, 16*).

Meningioma

The characteristic cytogenetic feature of cells from human meningiomas is the loss of 1 G chromosome (*6–8*), recently identified as #22 (*18, 19*). In a few tumors (5/70), part of the long arm of 1 G chromosome appears deleted; it is also a #22 (*20*). Since the chromosome abnormality in both CML and meningioma involves chromosome #22, a similar etiologic agent may cause both conditions. This suggestion might be incorrect, however, because published karyotypes from several meningiomas appear to have a normal #9 (*19*). The C chromosome that may be missing in cells from several of these tumors has been identified as #8 (*19*).

Burkitt's Lymphoma

A secondary constriction near the end of the long arm of 1 C chromosome has been observed in a variable proportion of cells from some cultured Burkitt's lymphoma cell lines (*21, 22*). A similar secondary constriction was present in ≈9% (2–25%) of normal human cells cultivated with *a*) lethally irradiated cells from Burkitt's lymphoma cell lines (*23*), or *b*) a filtrate of cells containing a herpes-like virus (*24*). The identity of the C chromosome carrying the secondary constriction has not been reported, but analysis of 1 photograph in which the chromosomes have faint bands [fig. 3 in (*21*)] suggests that it may be #11. The constriction was not seen in direct chromosome preparations of 4 Burkitt's tumors (*21*).

A consistent chromosome abnormality was observed only when cells from Burkitt's tumors and cell lines established from these tumors were examined with quinacrine fluorescence. An extra band of medium fluorescence was noted at the end of 1 chromosome #14 in every cell of 5 of 6 tumor biopsy specimens and 7 of 9 tumor cell lines (*25*). Whether the extra band was a duplication of a segment of #14 or the result of a translocation was not determined.

DISEASES WITH AN APPARENTLY VARIABLE CHROMOSOME PATTERN

Acute Myelogenous Leukemia (AML)

Chromosome abnormalities detectable by standard techniques have been identified in bone marrow cells from 30–100% (*26–28*) of patients with AML. In most cases, the modal chromosome number ranged from 40 to 60 or 90 to 100, and the abnormal pattern was highly variable. Groups C, D, E, and G had gains and losses of chromosomes more frequently than expected (*28*).

TABLE 1.—*Identification of specific chromosome abnormalities in various diseases*

Disease	Number of patients examined		Abnormality observed* (*30*)	Reference
	Total	With specific abnormality		
CML:				
Chronic phase	23	23	9q+, 22q− (Ph^1)	(*15*, *16*)
Acute phase	5	5	+8	(*15*, *16*)
	8	8	+ marker 17q	(*15*, *17*)
	5	4	Two Ph^1	(*15*, *16*)
Acute myeloblastic leukemia in females	3	2	45, X, t(8; 21)(q22; q22)	(*29*)
Polycythemia vera	6	6	20q−	(*16*, *33*)
Myelodysplasia	2	2	+8	(*36*)
Meningioma	10	8†	−22	(*19*, *20*)
	5	3	−8	(*19*)
Burkitt's lymphoma	12	10	14q+	(*25*)

*+: additional; −: absent; q: long arm; t: translocation. Consult text for further definitions of chromosome abnormalities.

† In 1 patient the entire short arm and one-third the long arm of #22 were deleted.

The new techniques have been used to identify patients whose cells show similar chromosome abnormalities. Marrow cells with a hypodiploid chromosome number were found in 3 female patients with AML. Cells from 2 of these had an identical complex karyotype (*29*), which suggests that chromosome abnormalities in this disease may not be entirely random. Cells from both patients had 45 chromosomes and were lacking an X. In addition, a portion of the long arm of 1 #8 was translocated to 1 #21; this produced 2 translocation chromosomes, 1 of which resembled a #16 and the other, a D chromosome. The breaks in 8q and 21q appeared to occur in the same bands in both patients. The translocation was assumed to be reciprocal. The karyotype can be expressed as 45,X, t(8;21)(q22;q22) (*30*). The marrow of both patients showed a predominance of myeloblasts, and both had a rapidly fatal course.

Polycythemia Vera (PV)

Variable chromosome abnormalities were observed in bone marrow cells from approximately 22% of patients with PV (*31*), whether or not they were treated. The arm of 1 F chromosome (F?−) was deleted in cells from $\approx 15\%$ (*31*) of patients with PV (more than half of all patients with abnormalities). Three patients whose cells contained normal F chromosomes on initial examination showed the F?− chromosome after treatment with ^{32}P, X-rays, or busulfan. This suggests that the F chromosome of patients with PV has a site particularly susceptible to the action of mutagenic agents. Alternatively, the F?− clone of cells may have been present before treatment, but in such small numbers that the clone was not detected until later in the disease. Abnormalities of F chromosomes also have been reported in some patients with idiopathic sideroblastic anemia (ISA) (*32*). Both diseases have increased risk of termination in acute leukemia.

In Giemsa banding (*33*) and quinacrine fluorescence (*16*) studies, the F?− in PV has been identified as a #20 chromosome that has lost approximately half to two-thirds of its long arm (20q−); whether the material missing from the #20 has been translocated to another chromosome is uncertain. Sometimes the short arm of the same #20 appears slightly smaller than its homologue (*16*). Patients whose cells show the 20q− chromosome in the polycythemic phase keep this abnormality, and the cells show additional abnormalities in the acute leukemic phase (*16*, *31*). Cells from 1 patient with leukemia had 2 20q− chromosomes (*16*), a phenomenon analogous to the double Ph^1 in the acute phase of CML.

Myelodysplasia

The significance of the chromosome abnormalities in marrow cells of some patients with other hematologic disorders is uncertain. Aneuploidy is an early change in some patients who later develop leukemia (*34*); in others, the karyotypic change is stable and may persist for several years. I studied 1 such patient with thrombocytosis, who had 2 extra C chromosomes in some marrow cells for 8 years without any disease progression (*35*). It is possible that all patients with aneuploid cells in the marrow will develop leukemia if they live long enough, and that our period of observation is too short compared with the time of evolution of the disease. Alternatively, various chromosomes may be involved because of the action of different etiologic agents, and these chromosomes may differ in their prognostic significance.

The abnormality most frequent in marrow cells of patients with aneuploidy is 1 or 2 additional C chromosomes, regardless of whether the diagnosis is leukemia, PV, or a "nonmalignant" hematologic disorder. Preliminary evidence from 5 patients with CML in the acute phase (*15*, *16*) and from 2 with marrow aplasia and granulocytopenia (*36*) suggests that chromosome #8 may be involved more frequently than expected by chance. In 3 of 5 meningiomas, 1 #8 chromosome was missing (*19*). Trisomy #8 is the only constitutional chromosome abnormality (i.e., all cells are abnormal) that involves a C group trisomy identified thus far (*37*). This may indicate that abnormalities of the genetic material carried on #8 are less deleterious to the individual, and that excesses or deficiencies can be tolerated as somatic mutations more readily than other types of chromosome imbalance. On the other hand, some genetic information carried on #8 may be important in the development of diseases in which #8 is abnormal because of excess, deficiency, or translocation of its chromosome material.

Consistent chromosome rearrangements have been observed in 3 diseases discussed here. A specific translocation involving #9 and 22 is thought to be associated with CML (*15*). Whether a specific translocation also occurred in cells with the 20q− chromosome (PV) or the 14q+ chromosome (Burkitt's lymphoma) has not been determined. It is presently impossible to decide whether some agent causes a particular chromosome change simultaneously with the tumor or premalignant state, or whether chromosome changes occur spontaneously and only the chance occurrence of a specific chromosome change leads to tumor formation. Careful cytogenetic analysis of cells from many neoplastic and preneoplastic conditions should help distinguish these possibilities as well as others.

SPECIFIC CHROMOSOME ABNORMALITIES INDUCED IN ANIMALS AND IN VITRO

Nonrandom chromosome abnormalities occur in some animals exposed to chemical and viral mutagenic agents; e.g., trisomy for the largest telocentric chromosome in rats was observed in leukemia (*38–40*) and sarcomas (*41*) induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) and 6,8,12- and 7,8,12-trimethylbenz[*a*]anthracene; trisomy for a metacentric chromosome was less frequent (*38*, *40*). A different, but very consistent karyotypic pattern was seen in sarcomas induced in the rat by Rous sarcoma virus (RSV) (*42*). The pattern of karyotypic evolution involved additions of 1 medium-size telocentric chromosome, followed by 1 medium and 1 small subtelocentric chromosome. Chromosomes most frequently abnormal in DMBA-induced tumors were rarely abnormal in RSV-induced tumors, and vice versa. *The sarcomas produced by DMBA and RSV were histologically indistinguishable.*

A similar phenomenon was observed in the Chinese hamster. One or more additional chromosomes belonging to 3 pairs (#5, 6, or 10) were found in most sarcomas induced by RSV (*43*), whereas #11 was the most frequent addition in cells from DMBA-induced sarcomas (*44*) in the same inbred Chinese hamster strain.

Even in experimental animals, some chromosome variability was present in tumors induced with a single, known oncogenic agent. It may be significant, however, that chromosome variability was less in inbred (20–30%) than in randombred (about 50%) animals.

Various in vitro systems also have been used to study the association of chromosome abnormalities with malignancy. Harris, Klein, and coinvestigators (*45*, *46*) found that hybrid cells, formed by fusion of malignant and nonmalignant cells, produced an increasing number of tumors in vivo as they lost certain specific, but as yet unidentified, chromosomes. The authors concluded that malignancy behaves like a recessive trait. Quinacrine fluorescence has been used to compare the karyotype of a nonmalignant L cell line and its malignant derivative (*47*). The significance of the chromosome differences between the 2 cell lines is not known but can be determined by analysis of additional cell lines.

Other investigators proposed that malignant cell transformation and its reversion are controlled by the balance between genes for expression and genes for suppression of malignancy (*48*). The chromosomes of golden hamster cells transformed with a chemical carcinogen (dimethylnitrosamine) have been analyzed with one of the new Giemsa banding techniques (*10*). The transformed cells had a consistent chromosome pattern different from that observed in less malignant revertant cells (*49*). These cells produced tumors when they were injected into animals; the cells from these tumors also showed a consistent karyotype. The close association of gains or losses of particular chromosomes with the malignant or nonmalignant character of the cells supports the above proposal.

MECHANISMS PRODUCING CHROMOSOME ABNORMALITIES SPECIFIC FOR DIFFERENT ETIOLOGIC AGENTS

One mechanism that could account for the chromosome differences observed with different oncogenic agents is suggested by an observation of Sugiyama (*50*), who noted that 2 regions on the long telocentric chromosomes, frequently present in a trisomic state in DMBA-induced rat leukemia, were specifically vulnerable to the chromosome-damaging action of DMBA. These same regions were late replicating and heterochromatic. It has been known for some time that such regions show preferential susceptibility to mutagenic agents (*51*).

A nonrandom effect on human chromosomes—the production of gaps and breaks at a specific site on chromosome #17—was observed in cells infected with adenovirus types 12 and 31, and to a lesser extent with type 18; infection with types 2 and 7 resulted in randomly distributed aberrations (*52*). A secondary constriction, indicating a heterochromatic region, was observed in the same specific site on #17 (*53*). In some circumstances, types 12, 18, and 31 are highly oncogenic in hamsters, type 7 is moderately oncogenic, and type 2 is nononcogenic (*54*). A similar nonrandom secondary constriction has been discussed in connection with Burkitt's lymphoma (*21–24*).

Various hypotheses concerning the role of genetic change and carcinogenesis are under investigation. In many autosomally recessive diseases in humans [including Fanconi's anemia (*55*), Bloom's syndrome (*56*), and ataxia telangiectasia (*57*)], a tendency to chromosome breakage and rearrangement is observed in in vitro cultures. Cells from patients with Fanconi's anemia (*58*), but not with Bloom's syndrome, have an increased susceptibility to viral transformation (*59*). Patients with these disorders have a greater than normal frequency of leukemia and other malignancies (*55–57*). The chromosome pattern in many of these individuals may be variable or normal. Hecht et al. (*60*) recently summarized data on the chromosome pattern of lymphocytic clones in 6 patients with ataxia-telangiectasia. All 6 had rearrangements involving a D chromosome, and 2 studied with the new techniques had a break in the long arm of #14.

The observation that at least part of the genome of oncornaviruses may be integrated in apparently normal host cells has led Huebner and Todaro (*61*) to propose the "oncogene" hypothesis—that the "oncogene," part of the oncornavirus genome existing as the DNA provirus, is included in the normal gene pool of all vertebrates and is transmitted vertically by the usual mechanisms of inheritance.

The relationship of the "oncogene" hypothesis to that proposed here is not clear. Possibly different "oncogenes" have different chromosome locations. Recent work, in which various genetic loci associated with susceptibility to murine leukemia virus (MuLV) are mapped to separate but specific chromosomes in inbred mice (*62–64*), supports this suggestion. The relationship of these gene loci to potential specific chromosome abnormalities in the mouse has not been determined. The establishment of linkage for a locus in a particular strain of mice carries no assurance that the same gene or its alleles will occupy the same site in the genomes of other mice (*64*). The mapping of genes was possible only with inbred mice and selected backcrosses of known genotype. This method is clearly not applicable to man.

CONCLUSIONS

The experimental data from animals are summarized as follows: 1) Genetic factors, which segregate in a Mendelian fashion and can be located on a specific mouse chromosome, control the susceptibility or resistance of a particular inbred mouse strain to infection with MuLV. 2) In a susceptible strain of rat or Chinese hamster, the particular chromosome abnormality observed depends on the mutagenic agent used in the experiment. 3) Histologically similar tumors caused by different agents have different karyotypic patterns.

The data collected about man are summarized as follows: 1) The relative importance of genetic and environmental factors in tumor production has not been determined. However, families as well as ethnic groups differ in the type and frequency of various tumors. 2) Cells from some types of tumors have a consistent chromosome pattern; Epstein-Barr virus is closely associated with, and has been proposed as, the etiologic agent for Burkitt's lymphoma (*65*), which is one of these tumors. 3) Cells from other diseases, including tumors, have a variable chromosome pattern; no specific etiologic agent has yet been identified for any of these diseases.

If the hypothesis proposed here is correct, one would expect to find that CML with the Ph^1 chromosome, meningiomas lacking chromosome #22, and Burkitt's lymphomas with the 14q+ chromosome have a specific etiologic agent different from the agent producing the chromosomally normal variant of each disease.

Furthermore, in the acute leukemias, PV, and other myeloproliferative disorders, one would expect to find a diversity of etiologic agents associated with their variable chromosome pattern. Within a disease category, however, careful karyotypic analysis should reveal patients with a similar karyotypic pattern whose disease is due to a single etiologic agent.

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ADDENDUM

Since this Editorial was first written, the evidence of nonrandom chromosome abnormalities in human leukemia and in other human cancers has become even more convincing (*1, 2*). Of particular importance is the presence of consistent translocations associated specifically with particular cancers; examples would be the 9;22 translocation in chronic myelogenous leukemia (*3*), the t(8;21) in acute myelogenous leukemia (*4*), the t(15;17) in acute promyelocytic leukemia (*5*), and the t(8;14) in Burkitt's lymphoma (*6*). Translocations involving the short arm of #1 have also been observed in patients with breast cancer (*7, 8*), and abnormalities of the long arm of #1 have been noted in various hematologic cancers (*9*) and in a number of carcinomas, such as those arising in the breast (*7, 8*), cervix (*10*), and ovary (*11*), among others. Many apparently viable rearrangements may result in a similar chromosome change; the aberrations that lead to trisomy for the same region of chromosome #1 (*9*) are an example.

The question of the relationship of specific chromosome abnormalities to etiologic agents is still unresolved. No evidence relates any particular chromosome change to any environmental agent. As indicated earlier, some chromosome changes are associated with a particular type of tumor, whereas others are seen in various neoplasms. We now have evidence that abnormalities of some chromosomes occur almost exclusively in cells of a particular type, e.g., translocations affecting #14 in lymphoid disorders.

One of the reasons for the uncertainty is the difficulty in definitely establishing the causative agent for a particular tumor. Although the Epstein-Barr virus (EBV) is associated with Burkitt's lymphomas, it cannot be stated that EBV causes them (*12*). Therefore, the observation that 8;14 translocation is present in EBV-negative (*6, 13*) as well as in EBV-positive tumors (*6*) cannot be used as evidence against the hypothesis. Since this is the only human tumor in which a specific agent has been putatively linked as causal, the reasons for our continued uncertainty regarding the correctness of the hypothesis should be evident.

Even in experimentally produced tumors in animals, when the inducing agent appears at first glance to be more obvious, the correct interpretation is not always clear. For example, we know that trisomy for #15 is regularly seen in mouse leukemias. This is true whether the leukemia occurs "spontaneously" in the AKR mouse (*14*) or is induced with exogenous virus (*15, 16*), with chemicals (*16*), or with radiation (*17*). That these chemicals and radiation induce the expression of endogenous virus is well-known. Therefore, all of these apparently different agents may act through the same effector mechanism, i.e., a recombinant virus.

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GUEST EDITORIAL **Guy R. Newell**[1]

JNCI 59:1581–1582, 1977

A Case for the Support of Treatment and Prevention Research in the Control of Cancer

Increasing publicity is being given to the assertion that 60–80%, perhaps even 90%, of all cancers are due to environmental factors and, therefore, are preventable. The approach seems so simple and logical that one could hardly argue against its implementation with all possible speed. In this context it is often noted that the National Cancer Institute (NCI) spends much less than 85% of its budget on environmental carcinogenesis, and the implication is that too much is spent on activities designed to find cancer cures.

The estimate that 60–80% of all cancers are from environmental causes is based primarily on differences in cancer incidence by site among various countries throughout the world. The overall difference between the highest and lowest occurrence is about 85% of all cancers. The interpretation that these differences are due to environmental influences is supported by studies of migrant populations who lose the characteristics of their native country when they move and assume living habits of their new country. This implies environmental rather than genetic factors. The reasoning is then extended by saying that, if these cancers are related to the environment, they are preventable. However, this is true only if the environmental factors are known and controllable. Removal of identified carcinogens depends on regulatory action, which is influenced by scientific, political, societal, and technical factors. Moreover, one should not assume that 85% of all cancers are related to specific known chemical carcinogens put into the environment by man, although this could well be part of the problem. To carry this oversimplification too far, as well as to ignore it, is dangerous.

Environmental carcinogenesis should be interpreted broadly rather than narrowly. The range of possible environmental influences is illustrated by the diversity of tumor types that might be affected and the different types of environmental influences possibly associated with each. One working definition of environmental carcinogenesis includes four general areas of activity.

First, efforts are directed toward the study of known environmental carcinogens: radiation (including ultraviolet light), tobacco, and chemicals (including drugs).

Second, screening for chemical carcinogens includes animal bioassay systems and efforts to develop in vitro assays. Since March 1977, we have been supporting work initiated by non-NCI investigators on 270 compounds. These are special studies, and the results will be reported by the investigators themselves rather than by the NCI Bioassay Program. Thus they are not under the "control" of NCI. The NCI Bioassay Program has standard test protocols for approximately 370 compounds, and results of these are to be reported by NCI. Items to be tested include food additives, pesticides, solvents, pharmaceuticals, biologics, and dyes.

Efforts to develop in vitro tests systems include 1) establishment of effective prescreen procedures to help in the creation of priorities for bioassays, 2) research into mutagenesis as it relates to carcinogenesis, and 3) research into mechanisms of action for identification of molecular targets and subsequent steps of the carcinogenic process.

Third, we have encouraged expansion of efforts to recognize patterns of environmental carcinogenesis. These activities include identification of persons and/or groups at high risk because of known exposure, geographic factors, ethnic background, family history, or other variables.

Fourth, we support and conduct research into the fundamental mechanisms of cellular processes of carcinogenesis, primarily through research grants, centers support, training grants and fellowships, and the modest support of construction. This research is critical because, due to the complex nature of our society and necessary institutions, some carcinogens may never be totally eliminated. Moreover, efforts to learn how to modify, block, or reverse the carcinogenic response in humans may not be rewarded for many years.

Even if all known and unknown sources of exposure to cancer-causing agents were eliminated immediately (which is impossible), the long latent period of 10–40 years for some cancers to develop after exposure would guarantee that millions of people would develop cancers into the 21st century. Obviously, we must do everything possible to treat and cure these patients.

Although control of most diseases throughout history has been achieved by prevention, this has not yet occurred for cancer. Cigarette smoking clearly contributes up to 40% of cancer in U. S. males and is increasing significantly among females. In British men, the figure is 50–60%. These include most lung cancers and a smaller proportion of cancers of the larynx, pharynx, esophagus, bladder, and pancreas. Thus a change in social habits based on information available today could significantly reduce cancer. Despite this, cancer incidence is increasing, especially among these sites. Specific known chemical carcinogens other than tobacco are estimated to contribute to less than 10% of the incidence.

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

Treatment research and its application are reducing cancer mortality among patients under 35 years of age due to improvements in patient care of acute leukemia, Hodgkin's disease, Wilms' tumor, osteogenic sarcoma, and others. Although this is a small segment of the population, knowledge gained from these successes is being applied to development of new treatment regimens for several more common types of cancer previously resistant to treatment, such as cancer of the breast, ovary, colorectum, head and neck, and lung.

Of profound significance has been a change in the conceptual approach to the treatment of cancer. In the past, the primary disease was usually treated by local eradication either with surgery alone or surgery and radiotherapy. If no residual disease was apparent, the patient was presumed to be free of disease and given no further treatment. Yet approximately 60% of all cancers would eventually recur (which strongly suggests that, although the primary tumor may have been completely removed, micrometastases remained), and these were then retreated with radiotherapy and eventually chemotherapy, probably with several drugs administered sequentially. Thus the disease was pursued but usually never caught.

Many oncologists now believe that most cancer patients have microscopic foci of metastatic disease and that initial combination of adjuvant chemotherapy with surgery and radiotherapy holds the best opportunity for future major successes in cancer treatment. It appears that this concept will be useful for several forms of cancer, including some of the more common types. Adjuvant chemotherapy has already proved effective for Wilms' tumor and osteogenic sarcoma. It may also be effective for Ewing's sarcoma. For breast cancer, the leading cause of death in women from cancer, indications are that major advances are forthcoming. For colorectal cancer, the second leading cause of cancer deaths in both men and women, adjuvant combination chemotherapy holds great promise. Opportunities for applying years of research toward major improvements in treatment have never been greater or more exciting.

Guest Editorial —— Jay H. Robbins, *M.D.* [1,2]

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Significance of Repair of Human DNA: Evidence From Studies of Xeroderma Pigmentosum

XP is a rare autosomal recessive disease in which all patients have accelerated chronic actinic degeneration of sun-exposed skin, including development of numerous sunlight-induced neoplasms (*1-3*). Some XP patients also develop neurologic abnormalities that are due to premature death of nerve cells (*1*). A decade ago Cleaver discovered (*4*) that cells from XP patients were defective in repairing UV radiation-induced damage to their DNA. Since then, numerous additional studies have confirmed that XP patients have defective repair of DNA damaged by UV and certain mutagenic chemicals (*1, 5-11*). XP is currently the only human disease in which inherited defects in DNA repair have been conclusively shown to exist. It is the purpose of this editorial to describe recent studies relating certain clinical manifestations of XP to the patients' defective DNA repair and to illustrate how such studies have contributed to our understanding of the consequences of physical and chemical damage to human DNA.

Despite the low incidence of XP, estimated to occur with a frequency of 1 (*12*) to 4 (*1*) per million births, established cell repositories[3] can provide cells from all 18 XP patients who were evaluated clinically at the National Institutes of Health (*1, 13*) as well as from several of the most important and unusual XP patients from Europe and the Middle East whose cells were studied in detail at Erasmus University, Rotterdam, The Netherlands. Published studies of approximately 70 Japanese patients with XP (*14, 15*), together with numerous published reports on other XP cells, suggest that the literature may already contain data on as many as 150 XP strains. Photobiologic and cell-fusion studies show that there are at least the following seven confirmed genetic forms of XP: six excision-deficient forms designated complementation groups A through F (*1, 15, 16*) and the excision-proficient form (*17*) designated the "XP variant" (*18-21*). To understand the basic aspects of XP and to choose appropriate cell strains for study, one must understand the principles of this classification and some of the photobiologic and clinical features of each genetic form. Of particular importance is the heterogeneity of DNA repair-dependent processes recently found (*13, 22-24*) among strains within each of the most common excision-deficient groups (i.e., groups A and C). Furthermore, certain clinical manifestations are now known to be related to the overall capacity of a cell strain to repair its damaged DNA to a biologically functional state (*13, 22*). There is now substantial evidence (*13, 22, 25, 26*) for the hypothesis that the normal functioning of DNA repair processes prevents not only sunlight-induced neoplasia but also premature death of neurons in all healthy humans.

RELEVANT CLINICAL ABNORMALITIES ON SUN-EXPOSED SKIN

XP patients have excessive freckling on sun-exposed skin usually before 2 years of age (fig. 1) [for color photographs, cf. (*1*)]. There is evidence (*1, 27, 28*) that each XP freckle, as might be the case for freckles in normal persons, is a clone of melanocytes derived from a single melanin-producing cell that has undergone a UV-induced mutation. Sun-exposed XP skin also has hypopigmented areas that might also arise from mutations making the melanocytes unable to produce normal pigmentation. Many children with XP develop actinic keratoses and frank cutaneous cancers before they reach 10 years of age (*1*). Actinic keratoses are the most frequent, followed by basal and squamous cell carcinomas and malignant melanomas (*1*). These tumors in XP patients occur with the same relative frequencies as, and are clinically and pathologically indistinguishable from, those in persons without XP (*1, 29*). However, XP patients can develop hundreds of such tumors before they are 30 years old (*1*). The sunlight-exposed portions of the eyes of all XP patients develop conjunctivitis, and many patients develop neoplasms of the conjuctiva or cornea (*30*). All these XP pigmentation abnormalities and neoplasms might arise from somatic mutations induced by the UV radiation in sunlight that induces photochemical damage in the DNA of human cells (*31*). The mutation frequency per unit dose of UV is higher in XP cells than in normal cells (*32, 33*). Sun-exposed XP skin also shows marked atrophy, and in vitro studies of XP cells have shown them to be more readily killed by UV

ABBREVIATIONS USED: SV40=simian virus 40; UDS=unscheduled DNA synthesis; XP=xeroderma pigmentosum.

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radiation than normal cells (*13, 14, 22, 32*). Although all these XP abnormalities may be the result of photochemical damage in DNA, the mechanisms leading to the clinical manifestations are not fully understood.

DEFECTIVE UV-INDUCED UDS

Although UV radiation causes several types of damage to DNA (*34, 35*), the pyrimidine dimer is probably one of the most biologically significant types of UV damage (*36*). When DNA is exposed to UV radiation, covalently linked cyclobutane dimers form between adjacent pyrimidines on the same DNA strand (*1, 31, 34, 37–40*). In excision repair of DNA, the dimer-containing region is removed (probably by several sequential reactions involving at least an endonuclease and an exonuclease), the resulting space is then filled in by repair synthesis by a DNA polymerase with the use of the intact opposing strand as a template, and the newly synthesized portion is joined to the remaining part of its strand by a polynucleotide ligase. The steps through repair synthesis can be evaluated autoradiographically (fig. 2) by the measurement of the radioactive thymidine incorporated during UDS. UDS is DNA synthesis that occurs during phases of the cell cycle other than the scheduled S-phase (*1, 41*). Cultured dermal fibroblasts from all excision repair-deficient XP cells show a reduced rate of such UV-induced UDS. The excision defect is probably present in all XP cells capable of division and has been demonstrated with epidermal cells (*42–45*), conjunctival cells (*30*), peripheral blood lymphocytes (*17, 45*), and Epstein-Barr virus-transformed lymphocyte lines (*46*).

COMPLEMENTATION GROUP ANALYSIS

The autoradiographic technique for the determination of UV-induced UDS was first utilized in Bootsma's laboratory at Erasmus University (*47, 48*) to show by cell-fusion studies that at least three complementation groups were among excision-deficient XP strains. Subsequently, our laboratory found four complementation groups among the National Institutes of Health XP patients (*1, 49*). In a collaborative study between our laboratories (*16*) we had established by 1975 the existence of five complementation groups among the excision-deficient XP patients, and these groups were designated A, B, C, D, and E. More recently, the existence of another group, designated F, has been confirmed (*15*). When a fibroblast from one complementation group is fused with inactivated Sendai virus to a fibroblast from another group, each cell in the resulting binucleate cell (heterokaryon) provides the other with its missing gene product so that both nuclei perform more UV-induced UDS than either could perform when unfused. Thus the nuclei "complement" each other and acquire normal rates of UV-induced UDS (*1, 16, 49*) (fig. 3; binuclear cell *e*). Fusions between fibroblasts in the same complementation group show no complementation (*1, 16, 49, 50*) (fig. 3; binucleate cell *f*). Most likely each of the six complementation groups has a different deoxyribonucleotide sequence defective and all members of the same complementation group have the same nucleotide sequence defective. However, the defects within the nucleotide sequence of any given complementation group can differ except in the case of strains from the same kindred. This interpretation predicts heterogeneity of the repair defect (i.e., genetic polymorphism and multiple alleles) among strains from unrelated patients within a complementation group, and results from experiments of post-UV colony-forming ability (*13*) and from UV-induced sister chromatid exchanges (*23, 24*) are in accord with this hypothesis (*see* below).

Text-figure 1 shows results from one of the experiments in which we demonstrated for the first time that an XP patient with some neurologic abnormalities was in complementation group C. The control donor's irradiated mononuclear cells (text-fig. 1a) had a mean grain count of UV-induced UDS of 79.3 grains/nucleus (arrow), whereas the unirradiated cells (data not presented) had a mean grain count of only 0.85 grains/nucleus. When strain CRL 1333 was fused with a known group A (text-fig. 1b) or group D strain (text-fig. 1d) and the cells were irradiated, a population of binuclear cells (heterokaryons) was present whose nuclei had essentially the same frequency of grain distribution and mean grain count (81.0 and 84.0, respectively) as those of the control donor's irradiated mononuclear cells (text-fig. 1a), which indicated that complete complementation had occurred. No complementation occurred when strain CRL 1333 was fused to a known group C strain (text-fig. 1c). Thus strain CRL 1333 is assigned to complementation group C. The binuclear cells that gave rise to the populations of labeled nuclei having mean grain counts of 6.6 (text-fig. 1b) and 18.6 (text-fig. 1d) are homokaryons of the strains used in the particular fusions, and each homokaryon contains two nuclei from the same strain. The binuclear cells that gave rise to the population of labeled nuclei having a mean grain count of 10.6 (text-fig. 1c) are homokaryons of strain CRL 1333 or of strain XP9BE[4] as well as heterokaryons formed by the fusion of a cell from one strain with a cell from the other strain. These heterokaryons show no complementation, inasmuch as their nuclei are derived from strains in the same complementation group. The mean grain count of 10.6 (text-fig. 1c) was similar to the mean grain count (data not presented) for strain CRL 1333's irradiated mononuclear cells (10.1) and for its binuclear homokaryons (10.9) obtained on slides in this experiment in which cells from strain CRL 1333 were fused to each other. The data show that strain CRL 1333 has approximately 13% of the control donor's UDS rate and is, therefore, well within the range (10–20% of normal)

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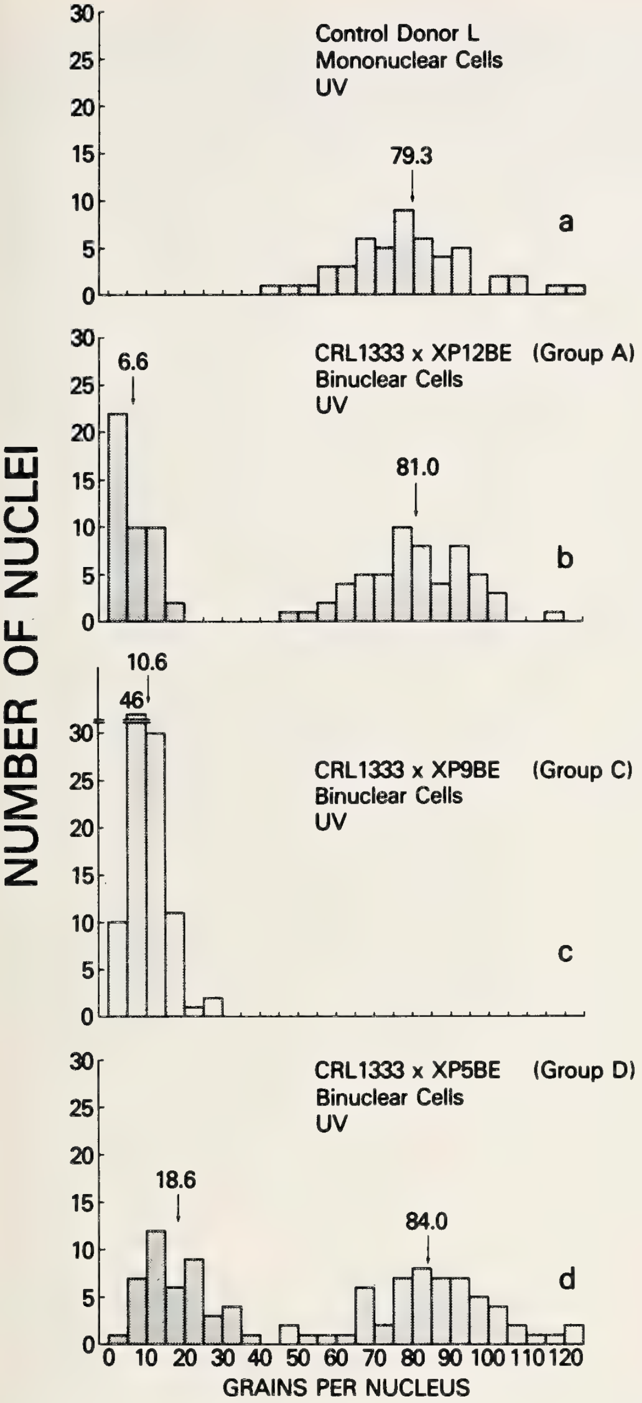
[4] In partial accordance with an international effort to standardize nomenclature of cell strains from patients with hereditary diseases, designations such as XP9BE will signify both the patient and fibroblast strains derived from the patient: XP, xeroderma pigmentosum; BE, Bethesda; LO, London; RO, Rotterdam; OS, Osaka. The number is that assigned to the particular patient in the city's XP patient series.



TEXT-FIGURE 1.—Histograms of autoradiographs showing that strain CRL 1333 is in group C. Methods as in legends to figs. 2 and 3 and (*1*, *49*); explanation and discussion are in text. (Data are from Robbins JH, Moshell AN, Andrews AD, et al: Unpublished data.)

previously reported for group C patients (*1*, *16*, *49*). The significance of this patient's complementation group assignment will be discussed.

FEATURES OF THE XP COMPLEMENTATION GROUPS

In the United States (*1*) and Europe (*5*) the most frequently found complementation group is group C. Only one group C patient appears to have the neurologic abnormalities of XP (*25*) (*see* below). Interestingly, no group C patient has yet been found among the 70 patients studied in Japan (*15*). Group A patients are the most common excision-deficient patients in Japan and are probably the second most common group in the United States (*1*) and in Europe (*5*). Most group A patients have very severe neurologic abnormalities that become manifest before 7 years of age, whereas other group A patients have few or no known neurologic abnormalities (*1*, *13*). The typical group A patient, with or without neurologic abnormalities, has from 0.4 to 1.3% of the normal rate of UV-induced UDS (*51*), but one patient, XP8LO, has 30% of the normal rate (*50*, *52*). XP patients in all five confirmed group D kindreds have neurologic abnormalities that generally become well manifested between 7 and 12 years of age (*13*). We (*1*, *16*, *49*, *53*) and others (*50*, *54*) have found these group D patients to have more UV-induced UDS than group C strains (i.e., 25–50% of the normal rate), although the Rotterdam investigators obtain a lower rate with one of our group D kindreds (*16*). Each of complementation groups B, E, and F is represented by a single kindred. Group B is of unusual interest in that the sole patient in this group has another rare autosomal recessive disease, Cockayne's syndrome (*1*, *55*), which is characterized by acute sun sensitivity, cachectic dwarfism, and neurologic and skeletal abnormalities (*55*–*57*). Although fibroblasts from patients with only Cockayne's syndrome have decreased post-UV colony-forming ability (*58*, *59*) and decreased host-cell reactivation of UV-irradiated adenovirus (*60*), their UV-induced UDS is normal (*59*), and they have no evidence of sunlight-induced skin cancers or pigmentation abnormalities (*56*, *57*, *59*, *61*, *62*).

BIOCHEMICAL AND PHYSICOCHEMICAL ABNORMALITIES IN XP CELLS

The primary inherited DNA repair defect has not been established in any form of XP. Probably such knowledge will be obtained only after purification to homogeneity of the appropriate enzymes and other factors involved in DNA repair in XP and normal human cells. The biochemical and physical abnormalities reported in XP cells are extremely complex for at least the following reasons: 1) Each agent that damages DNA, whether it is a physical (X-ray, UV) or chemical agent, produces more than one type of damage, and not all these types of damage are known; 2) abnormalities found in one XP cell strain may differ from those found in another strain, whether or not the strains are in the same complementation group.

The excision proficiency of the variant form of XP was first described in strain XP4BE (*17*) and was found in all his nucleated cells studied, i.e., peripheral blood small lymphocytes (*17*), dermal fibroblasts (*17*, *18*), epidermal cells (*42*, *43*), and even his tumor cells (*63*). Normal rates of excision repair have been confirmed in XP variants by studies of UV-induced UDS (*14*, *18*, *64*), the bromodeoxyuridine photolysis test (*1, 65*), removal of thymine dimers (*10*), and disappearance of endonuclease-sensitive sites (*66*). However, the variant had decreased host-cell reactivation of UV-irradiated adenovirus 2 (*19*). Such host-cell reactivation (i.e., repair) of UV-damaged, double-stranded DNA viruses that replicate in the cell's nucleus (*9, 15, 67–69*) and of UV-damaged, purified DNA of SV40 (*70*) has been shown to be defective in all XP patients whether excision-proficient or excision-deficient. These studies showed conclusively that the defective phenomena previously observed in XP cells were not due to abnormally increased UV damage to the XP cells (compared with the damage in normal cells), for only the virus was irradiated. The XP cells' inability to repair the UV-irradiated, purified SV40 DNA (*70*) and the low UV dose required to prevent reactivation of adenovirus in the most defective cells (*69*) indicate that the UV-induced damage which XP cells cannot repair normally is most likely pyrimidine dimers rather than DNA-protein cross-links.

XP variant fibroblasts were characterized further and found to have a severe defect in "postreplication repair" (*20, 71*), i.e., a defect in the ability to synthesize new daughter DNA immediately after UV irradiation. Such synthesis in the variant cells is sensitive to caffeine, but in normal cells is insensitive to caffeine. An interesting finding (*71*) was that the XP strains tested from groups A, B, C, and D have an intermediate defect in postreplication repair and some sensitivity to caffeine, whereas the group E cells show no detectable postreplication repair defect (*71*).

XP cells appear to repair single strand breaks (*72*) and probably most of the other biologically significant DNA damage induced by X-ray as well as normal cells (*73, 74*). However, one component of X-ray damage produced under anoxic conditions and reported to be repaired abnormally by XP cells (*75*) may resemble a form of UV-type damage that XP cells do not repair normally.

Other techniques have been used to study abnormalities related to DNA repair primarily in excision-deficient cells. Thymine dimers have been shown to be removed from the acid-insoluble fraction at a lower rate than normal (*76, 77*); repair replication has been shown to be deficient by centrifugation methods (*4, 34, 78–80*) and by the bromodeoxyuridine photolysis method (*65, 81*); the cells lose UV-endonuclease sites at a slower rate than normal cells (*66*); the cells show no detectable evidence of single-strand DNA breaks in the alkaline elution technique of Fornace et al. (*82*). It has been reported (*83*) that human cells have a light-dependent enzymatic repair process that monomerizes the thymine dimer, i.e., splits it so that the bases are restored to their preirradiation configuration without being excised. It has been further reported that XP patients (*84*) and their parents (*84*) are defective in such photoreactivation, but the significance, and even the existence, of this process in human cells have been questioned (*85*).

It has been reported (*86*) that group A and group D cells also have defects in another mode of excision repair than that described above, i.e., a defect in base excision repair. In base excision repair (*87*), damaged bases in the DNA are cleaved from the sugar in the DNA backbone, and the DNA strand is then incised by an apurinic-apyrimidinic site endonuclease. Other biochemical studies (*88*) have shown that extracts from some, but not all, XP strains are unable to excise pyrimidine dimers from chromatin but can excise them from purified DNA. Therefore, the repair enzymes of some XP strains may be unable to reach the DNA damage in their native chromatin.

As previously mentioned, XP cells are deficient in repairing DNA damaged by certain chemical mutagens-carcinogens (*1, 6, 7, 9, 11*). The *N*-acetoxy ester derivatives of aromatic amide carcinogens and the K-region epoxides of carcinogenic hydrocarbons kill and mutagenize XP fibroblasts more readily than normal fibroblasts (*32, 33*). Evidence indicates that different modes of DNA repair are required to repair the different types of damage caused by a single chemical carcinogen and to repair the different types of damage caused by different carcinogens (*6, 11, 87, 89*).

Although the chemical and other DNA repair-dependent abnormalities in XP are multiple, complex, and not currently understood, one may draw certain useful conclusions concerning the defects in XP and the patients' excessive UV-induced neoplasms and pigmentation abnormalities. All XP patients' skin fibroblasts have an abnormal susceptibility to the killing effects of UV radiation as demonstrated by their decreased ability to form colonies after UV irradiation in vitro (*13–15, 22*). In addition, all XP strains so far tested also have impaired abilities to reactivate UV-irradiated double-stranded DNA viruses (*9, 15, 69*) and purified viral DNA (*70*). Cockayne's syndrome patients who do not have coincident XP also have decreased post-UV colony-forming ability (*58, 59*) and decreased host-cell reactivation of UV-irradiated adenovirus (*60*) but do not have the tumors or pigmentation abnormalities of XP. Therefore, the defect(s) leading to increased sensitivity to the killing effects of UV and to decreased host-cell reactivation, as these parameters are currently measured, appear necessary but not sufficient phenomena to account for UV-induced neoplasia and pigmentation abnormalities. But the cutaneous abnormalities of sun-exposed XP skin develop whenever these defective parameters are present together with either decreased UV-induced UDS (e.g., XP group E and group F kindreds) or with abnormal "postreplication" repair (e.g., XP variants) or with both decreased UV-induced UDS and abnormal postreplication repair (XP groups A–D). Inasmuch as no human without such clinical evidence of premature, chronic solar skin damage has yet been shown to have one of the aforesaid combinations of defective DNA repair-dependent processes found in XP cells, probably the normal functioning of these processes protects normal humans from such premature solar damage.

Before concluding the discussion of DNA repair-dependent abnormalities in XP, I should emphasize that the processes by which photochemical and other damage to DNA leads to mutations (e.g., neoplasms and freckles) are still unknown. On the one hand, an "error-free" DNA repair process should not lead to mutations that result in cancer or freckles unless it is overwhelmed with DNA damage. An "error-prone" DNA repair process, on the other hand, would more likely result in heritable somatic mutations in the damaged cells' progeny. The normal human nucleotide excision repair process may be essentially error-free. In fact, there is evidence that in the excision-deficient XP cells, the residual UV-induced UDS of the excision repair process is error-free (*32, 33, 90*). Nevertheless, if the cells synthesize daughter DNA from parental DNA in which the excision repair has not been completed, heritable somatic mutations may result. Slow excision repair may also result in the presentation of photochemically damaged DNA to error-prone repair processes that would result in somatic mutations in either the parent or the daughter DNA. There are inducible error-prone DNA repair processes in bacteria (*91, 92*), and experiments are being performed (*93*) to determine whether they also exist in mammalian cells.

INCREASED UV-INDUCED SISTER CHROMATID EXCHANGES IN XP CELLS

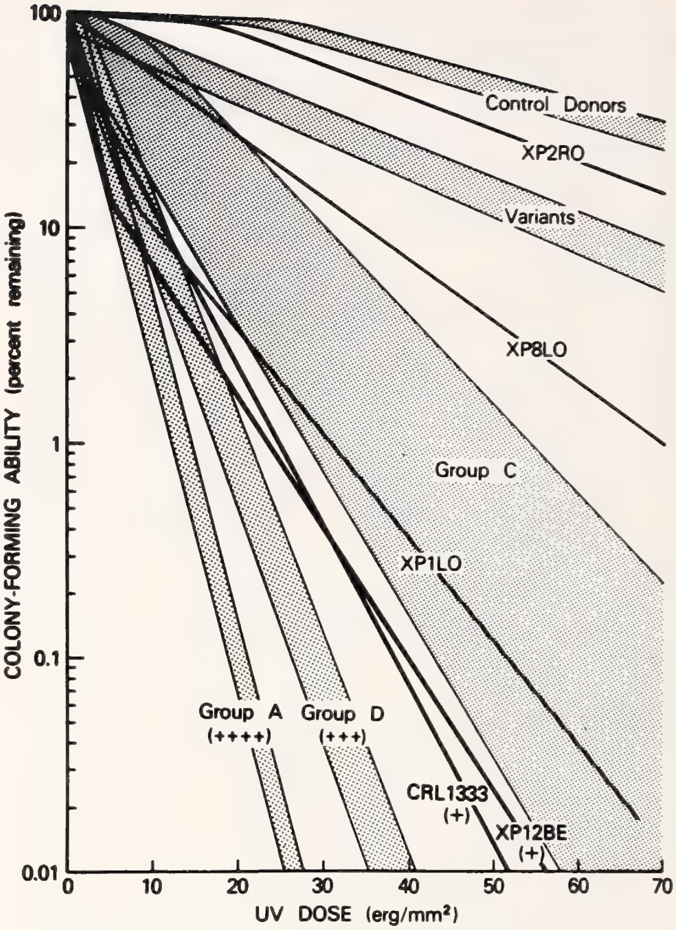
Sister chromatid exchanges have recently become amenable to appropriate study with the development of improved and accurate methods for their detection (*94, 95*). There are abnormalities in the number of sister chromatid exchanges in several diseases, all associated with the development of various cancers. For example, high frequencies of "spontaneous" sister chromatid exchanges are found (*96*) in phytohemagglutinin-stimulated cultured leukocytes from patients with Bloom's syndrome (*97*) and from skin cancer patients with histories of inorganic trivalent arsenic ingestion (*98*). Furthermore, the number of sister chromatid exchanges has been reported to be abnormally increased in untreated cells (*99*) from patients with the inherited disease dyskeratosis congenita and also in their cells (*100*) treated with trimethylpsoralen followed by 365 nm UV radiation, a combination believed sufficient to produce psoralen-DNA cross-linking photoadducts. Untreated XP cells show no abnormal number of spontaneous sister chromatid exchanges (*23, 24, 101–103*). However, after exposure of XP cells to 254 nm UV radiation, certain XP strains show much greater numbers of UV-induced sister chromatid exchanges than do normal cells (*23, 24, 101, 103*). In studies of this phenomenon in XP fibroblasts (*23*) and in XP lymphocyte lines (*24*) from excision-deficient XP patients and from XP variants, the following results were obtained (*23, 24*): Cells from all XP patients were capable of performing at least a normal number of UV-induced sister chromatid exchanges; cells from patients in groups A, C, D, and probably B showed abnormally large numbers of UV-induced sister chromatid exchanges; different strains within a group had significant differences in their abnormal number of UV-induced sister chromatid exchanges, but cells from XP siblings had identical increases. Although the precise molecular events involved in sister chromatid exchange formation are unknown (*104*), unrepaired DNA damage may cause the excessive UV-induced sister chromatid exchanges observed in XP cells. Of further interest is the recent finding (*105, 106*) that abnormally increased numbers of sister chromatid exchanges occur in XP fibroblasts exposed in culture to chemical carcinogens including those previously believed to be handled normally by XP cells, for example, methyl methanesulfonate or ethyl methanesulfonate, methyl-nitro-nitrosoguanidine, and ethylnitrosourea. Such sister chromatid exchange formation in XP cells constitutes a sensitive system for the detection of possible genetic effects of such chemicals on human cells (*105, 106*).

RELATIONSHIP BETWEEN POST-UV COLONY-FORMING ABILITY AND NEUROLOGIC ABNORMALITIES OF XP

Some XP patients develop neurologic abnormalities that first appear in infancy, childhood, or as late as the third decade of life, that usually worsen, and that are due to the premature death of central nervous system neurons in the absence of recognizable and specific histopathology (*1*). Therefore, as early as 1974, we have considered these neurologic abnormalities of XP to be the result of an abnormal aging of the human nervous system (*1*). The abnormalities include mental deterioration, microcephaly, sensorineural deafness, areflexia, choreoathetosis, ataxia, extensor plantar reflexes, spasticity, and a neuropathic electromyogram and muscle biopsy (*1*). Sufficient numbers of these neurologic abnormalities have been found in each of several group A patients (representing four kindreds) and in patients from all five confirmed group D kindreds to indicate that the patients' inherited deoxyribonucleotide sequences defining groups A and D, respectively, determine the occurrence of the neurologic abnormalities in these patients (*1, 25, 61*). Young children with XP who have all or most of these neurologic abnormalities, together with retarded growth and sexual development, are properly classified as having the De Sanctis-Cacchione syndrome (*1*).

However, our purported relationship between DNA repair and the premature death of nerve cells in XP patients had certain conceptual problems: Although patients in all five of the group D kindreds generally had a similar time of onset and type of neurologic abnormalities, some of the group A kindreds had severe neurologic abnormalities at an early age whereas others had few or no known neurologic abnormalities even as late as their fourth decade of life (*1*); no laboratory test had distinguished group A cell strains from patients with neurologic abnormalities from group A cell strains from patients with no known neurologic abnormalities. A second conceptual problem arose from our recent finding (*25, 61*) that one of the 13 known group C kindreds had a few neurologic abnormalities, whereas the other 12 had no evidence of XP-type neurologic abnormalities. We have recently performed post-UV colony-forming ability experiments (*13, 25, 61, 62*) that resolved these questions and showed for the first time a relationship between premature death of neurons and the capacity to restore damaged DNA.

Log-phase fibroblasts were irradiated with 254 nm UV light from a germicidal lamp, trypsinized, and replated at known densities. After 2-4 weeks' incubation the cells were fixed and stained in the dishes and then scored for colony formation. The colony-forming efficiency, defined as the number of colonies obtained divided by the number of cells plated, was determined for each UV dose. The post-UV colony-forming ability was calculated by the division of the colony-forming efficiency of the irradiated cells at a given dose by the colony-forming efficiency of that strain's unirradiated cells in the same experiment (*13*). Twenty-seven XP strains and five control donor strains were studied in over 100 experiments. From two to seven experiments were done with each strain.



TEXT-FIGURE 2.—Relationship between neurologic abnormalities of XP patients and the post-UV colony-forming ability of their fibroblasts. Explanation and discussion are in text. [Text-fig. 2 is fig. 4 in (*13*) which has been modified with the curve for strain CRL 1333 superimposed. Text-fig. is reproduced with permission from the *Proceedings of the National Academy of Sciences*.]

Text-figure 2 shows the results of these studies (*13, 25, 61*). The five group A strains with the lowest post-UV colony-forming ability are all from patients who had severe XP-associated neurologic abnormalities such as progressive mental deterioration, sensorineural hearing loss, ataxia, spasticity, choreoathetosis, and areflexia and in whom these abnormalities became clinically well manifested before the age of 7 years. The early onset of these numerous abnormalities is indicated in text-figure 2 as ++++. The group D strains tested, all of which have greater post-UV colony-forming ability than the aforementioned group A strains, are all from patients who had the same XP-associated neurologic abnormalities as the group A patients but in whom these abnormalities usually became clinically well manifested between 7 and 12 years of age and are, therefore, designated +++. The post-UV colony-forming ability curve for strain XP12BE of group A (*1*) is below that of the lowest non-neurologic group C curve (bottom line of group C zone) but for most of its long exponential portion is above the group D curves.

This strain was obtained from a patient, now 12 years old, when she was 7 years old. She had only two neurologic abnormalities, absent deep tendon reflexes and an abnormal electroencephalogram (*1*). Although the age of onset of these abnormalities is unknown, she has not developed any other neurologic abnormalities over the past 2 years. Her neurologic abnormalities are, therefore, among the mildest (+) of the patients with XP-associated neurologic abnormalities. The post-UV colony-forming ability curve for strain CRL 1333, from group C (text-fig. 1), is located close to that of strain XP12BE. Strain CRL 1333 was from a 17-year-old woman with XP who was reported to us to have only a few neurologic abnormalities, viz., mental retardation and microcephaly. Her post-UV colony-forming ability curve, like the curve for XP12BE, is significantly below the lowest group C curve that forms the lower border of the non-neurologic group C zone but significantly above the curve forming the uppermost border of the group D zone. Thus the post-UV colony-forming ability curves for these 2 patients traverse what has been called the post-UV "transition zone" (*61*) and is situated between the neurologic group D zone and the non-neurologic group C zone. Within this zone we might expect, and apparently have found, post-UV colony-forming ability curves from XP patients with only a few clinically apparent neurologic abnormalities of XP in their second decade of life.

Almost all of the post-UV colony-forming ability curve of group A strain, XP1LO, is within the zone of the group C curves, whereas most of the curve for the highest group A strain, XP8LO, is above that of any group C strain tested. Neither XP1LO (currently 39 yr old) (*107*) nor patient XP8LO (currently 6 yr old) (*50*) has any known XP-associated neurologic abnormalities. Similarly, none of the eight group C patients (representing seven kindreds), the two XP variants, or the group E patient (XP2RO), most of whom are already adults and whose curves are represented in text-figure 2, has any known XP-associated neurologic abnormalities.

The differences in post-UV colony-forming ability among the XP strains and between the XP and control donor strains reflect the different capacities of the cells to repair their UV-damaged DNA to the biologically functional level required to complete repeated cycles of cell division. That differences between XP strains' post-UV colony-forming ability result only from differences in their inherited mutations at the DNA repair loci is supported by the findings that, among the four XP sibling pairs studied, post-UV colony-forming ability was identical among strains from siblings (*13*).

It was important to determine whether the same relative sensitivities to UV shown by fibroblasts in our post-UV colony-forming ability experiments could be demonstrated with a slightly different test of cell killing (staining with trypan blue) and with a different cell type. Accordingly, we prepared lymphocyte lines from many of our XP patients by transforming their peripheral blood B-lymphocytes with Epstein-Barr virus. We had previously shown that XP patients' lymphocyte lines manifested the same DNA repair-dependent abnormalities as their fibroblasts, i.e., increased sensitivity to UV radiation (*108*), decreased rates of UV-induced UDS (*46*), and increased UV-induced sister chromatid exchanges (*24*). Results with this system have so far indicated the same relative sensitivities to UV radiation as those obtained with the post-UV colony-forming ability of the XP patients' fibroblasts as the following relative survivals of the lymphocyte lines indicate: normal control donors > group E (XP3RO) > XP variant (XPPHBE) > high group C (XP2BE; XP8BE; XP9BE) > low group C (XP1BE; XP3BE) > group A patient with only a few neurologic abnormalities (XP12BE) > group D (XP17BE) > severe neurologic group A (XP20S) (Andrews AD, Moshell AN, Robbins JH; Moshell AN, Newfield SA, Andrews AD, et al: Unpublished observations).

The fibroblast and lymphocyte survival results suggest that different kindreds within a single complementation group (e.g., A or C) display genetic polymorphism, i.e., have different inherited mutations in their DNA repair gene loci. The results also suggest that XP patients with neurologic abnormalities have DNA repair processes so defective that neuronal DNA, perhaps damaged "spontaneously" or by endogenous chemicals, is not repaired to the extent required for neuron survival. Such damaged neuronal DNA apparently requires the same process(es) for its repair as DNA damaged by UV radiation. At least three discrete deoxyribonucleotide sequences, those defining DNA excision repair complementation groups A, C, and D, determine the occurrence of the neurologic abnormalities in XP patients. Considerable evidence suggests that the nucleotide sequence defined by the group A defects and that defined by the group D defect(s) are distinct and do not overlap, inasmuch as fusing a group A fibroblast with a group D fibroblast results in a heterokaryon with fully restored UV-induced UDS (*1, 16, 49*) and host-cell reactivation (*109*). Furthermore, the relevant group C nucleotide sequence appears discrete from the sequences defining groups A and D, inasmuch as fusing the neurologic group C patient's strain (CRL 1333) with a group A (text-fig. 1b) or group D strain (text-fig. 1d) results in heterokaryons with fully restored UV-induced UDS.

Our XP data can be extended logically to the following hypotheses (*13, 25, 61, 62*). The damage induced in the XP cells by the UV radiation requires the same process(es) for its repair as the currently unknown damage we postulate must occur in the DNA of the central nervous system of both XP and normal individuals during embryonic and postnatal life (*1*). The XP neurons are not repaired as efficiently as normal neurons. As further damage occurs, the XP neurons die prematurely when their genetic information is reduced below the critical level required for biologic function. Even though human neuronal DNA does not replicate after the second postnatal year, the functional integrity of the DNA of these post-mitotic neurons is essential for the survival of the cells. Therefore, damaged neuronal DNA could be involved in the pathogenesis of other degenerative diseases of the human nervous system as well as in "normal aging" of that system. The loss of functional integrity of neuronal DNA can be the result of inherited defects in DNA repair processes such as in XP. Other inherited diseases, such as Cockayne's syndrome (*55–57*), ataxia telangiectasia (*8, 110, 111*), and Fanconi's anemia (*112*), can also have degeneration and/or other abnormalities of the nervous and other organ systems, and cells from these patients have been shown to be abnormally sensitive to certain physical and/or chemical mutagens. Thus Cockayne's syndrome cells are abnormally sensitive to UV radiation (*58, 59*), ataxia telangiectasia fibroblasts [(*113, 114*); Andrews AD, Barrett SF, Robbins JH: Unpublished observations] and lymphocyte lines [(*115*); Moshell AN, Newfield SA, Robbins JH: Unpublished observations] to γ-rays and possibly to certain chemicals (*8, 11, 116*), and Fanconi's anemia cells to the DNA interstrand cross-linking properties of certain chemicals (*117, 118*). Whether or not these diseases are ultimately convincingly shown to be caused by inherited primary defects in DNA repair, it is possible that their nervous system and other clinical abnormalities are due to DNA damage caused by endogenous chemicals or physicochemical events to which their cells are abnormally sensitive in a manner analogous to the postulations concerning death of neurons in XP (*1, 13, 61, 62*). It is of interest that patients who have ataxia telangiectasia (*8, 12, 110, 111*) or Fanconi's anemia (*12, 112*) have increased incidences of malignant neoplasms in various organs.

RELATIONSHIP BETWEEN POST-UV COLONY-FORMING ABILITY AND ACUTE SUN SENSITIVITY IN XP PATIENTS

Among the 18 XP patients we have evaluated clinically (*1*), we have been unable to relate the degree of the patients' accelerated solar skin degenerations to any in vitro tests of DNA repair. Any such correlation that might exist would be obscured by the absence of accurate quantitation of the patients' previous sun exposures. However, we have found a correlation between the patients' post-UV colony-forming abilities and the presence or absence of a history of acute sun sensitivity (skin blistering and/or severe erythema after a sun exposure, which would not produce such results in normal individuals). XP patients whose cells had the lowest post-UV colony-forming abilities had histories of acute sun sensitivity, whereas patients with higher post-UV colony-forming abilities had never experienced such acute reactions (*13, 26*). Thus the acute sun sensitivity experienced by some XP patients apparently results from excessive killing or damage of cells in their skin as a result of inadequate DNA repair.

PERSPECTIVES

XP is the first human disease conclusively shown to be caused by inherited defects in processes required for the repair of damaged DNA. When the molecular defects and abnormal DNA repair-dependent processes of XP are more completely elucidated, we shall learn more about how damage to DNA results in somatic mutations that can cause cancer in humans. Methods for the study of the DNA repair defects in XP cells have encouraged and facilitated the search for defects in DNA repair in other inherited human diseases (*58-60, 71, 99, 100, 113, 114, 117-119*) that are characterized by increased incidences of cancer and/or congenital malformations (*56, 57, 97, 110-112*). It can be confidently expected that future studies of DNA repair processes and of the effect of damage to DNA in XP and other diseases will eventually provide a better understanding of carcinogenesis, organogenesis, and cell function and survival. Such studies will also lead to methods for the identification of heterozygous carriers of these autosomal recessive mutations who may be at high risk for developing certain cancers and who may comprise a significant proportion of patients with such cancers (*12, 120*). However, as has been shown to be the case with XP, progress in the study and understanding of the biologic significance of abnormal DNA repair processes in human beings will continue to require appropriate detailed knowledge of the patients' clinical abnormalities.

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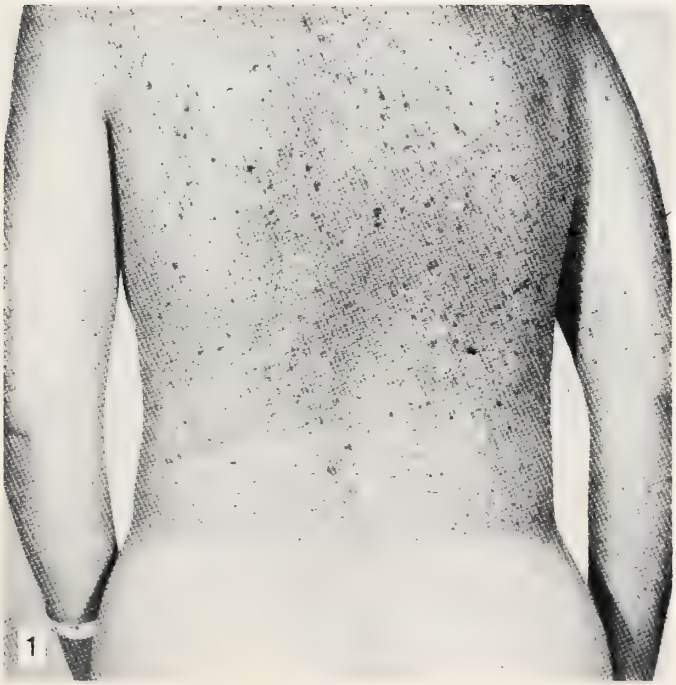
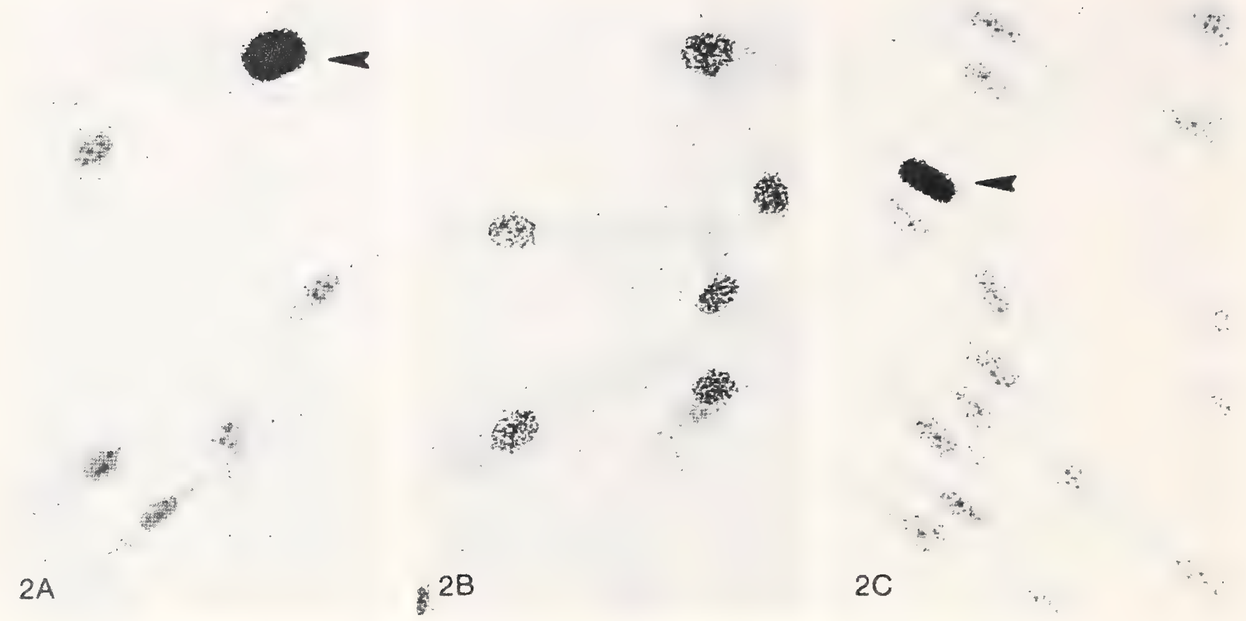


Figure 1.—Areas of skin protected from sunlight have not developed pigmentation abnormalities, keratoses, or cancers.



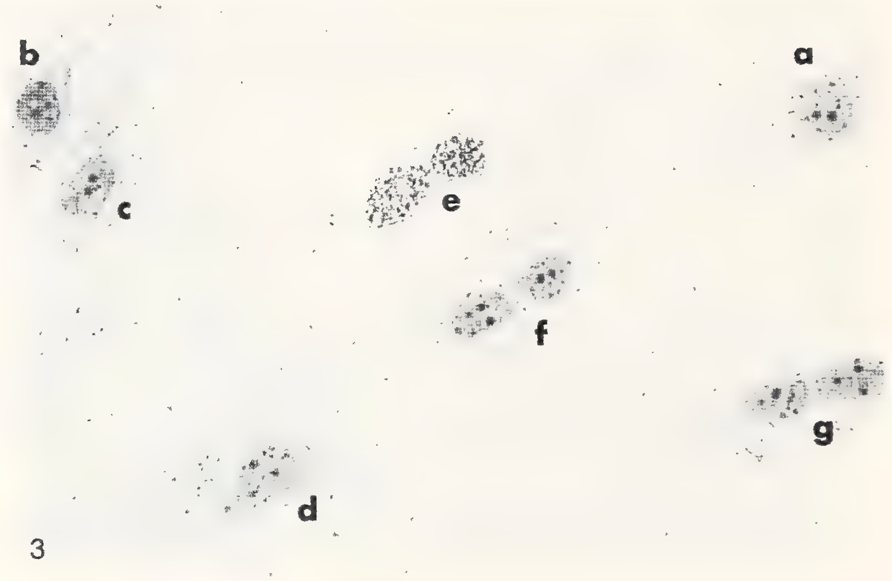


FIGURE 2.—Autoradiographs of normal and XP fibroblasts. A: Normal donor's nonirradiated cells after 3-hr incubation with tritiated thymidine. The cell, which is "heavily labeled" (*arrowhead*), was in scheduled (S-phase) DNA synthesis. The other cells are unlabeled. B: The normal donor's UV-irradiated cells after a 3-hr postirradiation incubation with tritiated thymidine. All cells (except for heavily labeled S-phase cells not shown) are "lightly labeled" owing to the UV-induced incorporation of tritiated thymidine during DNA repair synthesis. C: Irradiated cells of strain XP2BE after a 3-hr postirradiation incubation with tritiated thymidine. All cells other than the heavily labeled cell in S-phase synthesis (*arrowhead*) are lightly labeled, owing to repair synthesis, but the amount of labeling and, therefore, of repair synthesis is considerably less than that of the normal donor's cells. Fibroblasts irradiated in saline with 150 ergs/mm² of UV light from a General Electric germicidal lamp (No. G15T8), emitting predominantly 254 nm UV light. The cells were incubated for 3 hr with tritiated thymidine in 37° C air:CO_2 (95%:5%) incubator. Then they were washed with phosphate-buffered saline, fixed in a glutaraldehyde-cacodylate solution, and prepared for autoradiography (emulsion, NTB-3; exposed time, 1 wk). [Figs. are from (*1, 46*). They are reproduced with permission from the *Annals of Internal Medicine.*] Acid hematoxylin. × 300

FIGURE 3.—Autoradiograph of unfused mononuclear fibroblasts (a to d) and of binuclear fibroblasts (e to g) in a Sendai virus-treated culture containing cells from patients XP1BE (group C) and XP12BE (group A). Cells were exposed to 150 ergs/mm² of UV light and treated as described in the legend of fig. 2. The binuclear cell with numerous grains over its nuclei (e) is a heterokaryon whose nuclei have complemented each other, inasmuch as both nuclei have a normal or nearly normal amount of UV-induced tritiated thymidine incorporation, compared with the incorporation in the normal cells in fig. 2B. The other binuclear cells (f and g) are homokaryons, and their nuclei have no more incorporation than the nuclei of the unfused mononuclear cells (a to d). [Fig. is from (*1*). It is reproduced with permission from the *Annals of Internal Medicine.*] Acid hematoxylin. × 420

PART II: LABORATORY STUDIES

The Nature of Cancer

General Cancer Biology

Chemical and Physical Carcinogenesis in the Laboratory

Immunobiology of Cancer

Immunodiagnosis of Cancer

Immunotherapy of Cancer

Tumor Virology

Pathologic Aspects of Cancer

GUEST EDITORIAL

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JNCI 46:III–VII, 1971

The Protovirus Hypothesis: Speculations on the Significance of RNA-Directed DNA Synthesis for Normal Development and for Carcinogenesis

THE NEOPLASTIC phenotype usually persists in progeny of cancer cells. An understanding of the mechanism of this persistence appears necessary to understanding the etiology of the cancer. Theories of persistence include genetic theories such as those involving somatic mutations or viruses, and epigenetic theories such as those involving embryonic arrests and activation. The oncogene theory (*1*) appears to combine viral and epigenetic theories by postulating that neoplasia results from the activation of a vertically transmitted provirus. However, this oncogene theory is basically an epigenetic theory, since it postulates a stable change in phenotype due to a switch in a regulatory system.

Recently, I have suggested a new genetic hypothesis which combines elements of somatic mutation and viral theories (*2, 3*). This protovirus hypothesis proposes apparent vertical transmission of the information for cancer, even though the germ line does not contain this information on its chromosomes (proviruses or oncogenes) or off its chromosomes (virions). The germ line is postulated to contain in its chromosomes the potential for genetic evolution by the somatic cells that may lead to the *de novo* formation of the information for cancer. Changes leading to cancer are not the normal roles of this genetic system. Normally the protoviruses from the germ line evolve in the somatic cells to differentiate the genomes of these cells by the creation of new DNA.

This apparent vertical transmission of the information for cancer differs from classical vertical transmission which involves the whole virus as an infectious particle in, for example, the milk or egg. It also differs from transfer of the DNA provirus of an RNA tumor virus, since in the protovirus hypothesis only the potential for genetic evolution of the information for cancer is transmitted in the DNA.

The earlier DNA provirus hypothesis, from which the protovirus hypothesis was derived, stated that replication of RNA tumor viruses involved a DNA intermediate and that permanent establishment in a normal cell of the genetic information for cancer required information transfer from viral RNA to DNA (*4*). Once this information transfer from RNA to DNA had occurred, further transfer of information from RNA to DNA was not required for the maintenance or the expression of the neoplasia. The DNA provirus hypothesis did not elucidate how the establishment in a cell of this new genetic information led to the neoplastic state.

Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors will welcome suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

Normal Development

In speculating about the origin of viruses with this special mode of information transfer, RNA→DNA, I felt that RNA→DNA information transfer might play a role in normal organisms. Such reverse transcription could provide a mechanism for variability in the genome of somatic cells without disrupting the stability of the germ line. In the germ line only the usual modes of information transfer occur, that is DNA→DNA, DNA→RNA, and RNA→protein. Variation of expression occurs by control of the rate of these reactions, but variation of the DNA is supposed to occur only by random mutation followed by selection. The protovirus hypothesis states that somatic cells, in addition, use the RNA→DNA mode of information transfer, and new DNA sequences are formed by this process during the lifetime of a single organism.[1]

An organism needs to identify cells in a stable way, so that one cell is identified as a retinal cell at a particular position, and another cell is committed to make antibody to a particular antigen. The most stable storage place for such information appears to be the cellular DNA. RNA→DNA information transfer in somatic cells would provide a mechanism for stable differentiation of DNA. Other mechanisms are possible, that is stable cycles of the type discussed by Monod and Jacob (*5*) or classical DNA episomes. The protovirus mechanism might be preferred to cycles because it involves a structure rather than a dynamic process. The protovirus mechanism might be preferred to classical DNA episomes because it does not require excision of DNA for information transfer. However, it is possible that all of these mechanisms exist.

The process of protovirus transfer might work as follows (text-fig. 1). A region of DNA in cell A serves as template for synthesis of an RNA which is transferred to cell B. In cell B, a new DNA is made by an RNA-dependent DNA polymerase, using the transferred RNA as template. The new DNA then integrates into the DNA of cell B. This integration could be next to the homologous DNA or at a different place. In either case, cell B would differ from cell A, which remains unchanged. This process could be repeated. If the new DNA in cell B was integrated in some new place, it might act as a template for RNA from itself and from a neighboring region. This RNA in turn could be transferred to cell C, transcribed into DNA, and integrated into the genome of cell C. Cell C would then differ from cell B and from cell A. These processes could continue and could involve a cell more than once. They would lead to the formation of duplications, tandem or distant, and possibly replacements.

In addition, there might be times when the RNA→DNA information transfer takes place only in cell A, with or without concomitant integration, thereby leading to gene amplification (*6*). However, if, as I believe (*3*), there is great genetic instability in this type of information transfer, variants would appear and wholly new DNA sequences could be formed.

There would be regions in the genome predetermined for this type of transfer. The specificities of the appropriate polymerase and integration systems would select these regions on the basis of their base sequences. The time and extent of protovirus transfer would be controlled by the availability of the polymerase and integration systems, and the absence of possible inhibitory systems, especially nucleases. Selection of randomly appearing variants could also be through the properties of the polymerase, integration, and, possibly, inhibitory systems. This selection also would involve selection of cells altered by the presence of new protovirus DNA. These selection processes would allow random variations to persist and to become amplified. Together these processes could lead to differentiation among the genomes of the somatic cells in an organism.[2] In the development of an organism, information transfer from DNA→RNA→DNA would allow variability and

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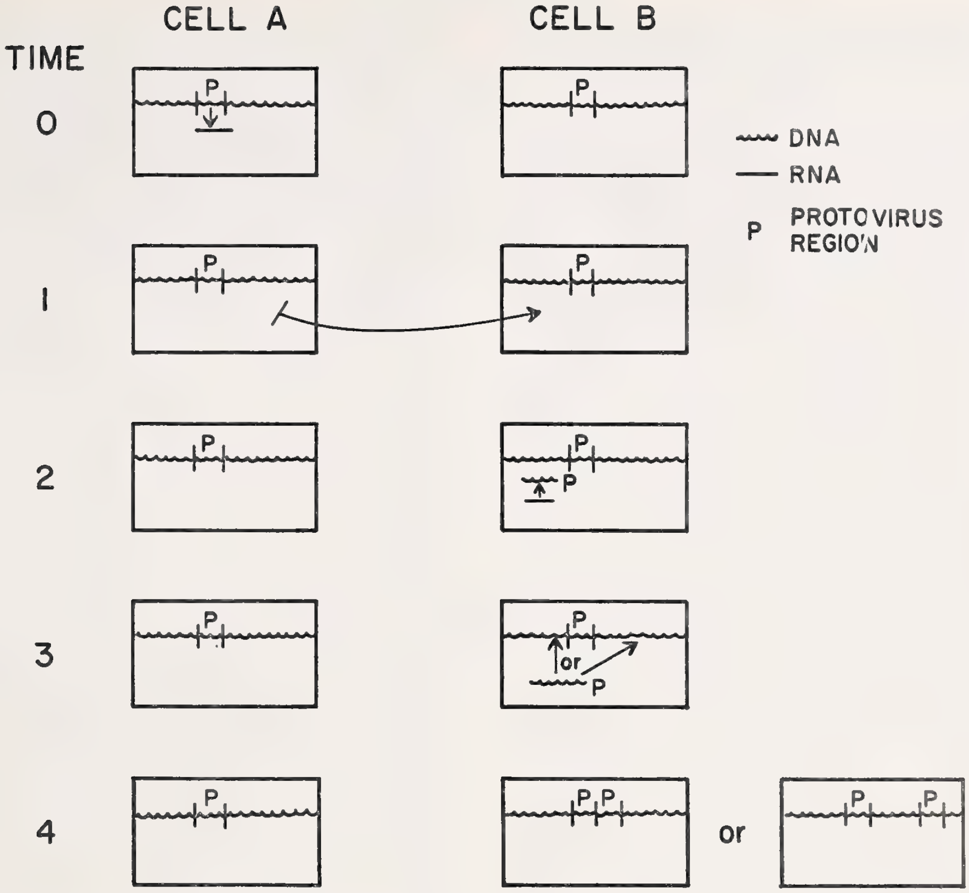
[1] Information transfer from RNA to DNA could occur in the germ line without affecting progeny individuals if this DNA was not integrated. Strictly speaking, only integration of such DNA would usually be forbidden in the germ line.

[2] The successful experiments on nuclear transplantation from somatic cells to eggs (*7*) do not invalidate the protovirus hypothesis. There are two aspects of this hypothesis which can lead to reversibility. One is crossing-over to remove a duplication, as seen in the bar region of Drosophila (*8*). The other is epigenetic controls, which could inactivate regions of new genetic information in embryonic cells. These hypotheses might also encompass the cases of reversibility of the malignant phenotype (*9*).



Text-figure 1.—One step in protovirus evolution. Two separate cells, A and B, are shown at five successive times, 0–4. Cell A transfers a protovirus-derived RNA to cell B at time 1. DNA derived from this RNA is integrated into the DNA of cell B at time 3.

amplification; information transfer from DNA→DNA would allow stability and storage.

The normal physiological evolution of the protovirus-derived DNA's would fall within a pattern predetermined by the rest of the cell genome and by the state of the cell and of the developing organism. Integration of protovirus-derived DNA, specifying some polymerases, for example, next to a region controlling membranes or other aspects of the cell surface could affect surface specificities of the cell. Integration next to a region controlling cell multiplication could affect multiplication control of the cell. Continued evolution along these lines could put together in a contiguous region of the chromosomes the information necessary for formation of an enveloped virion.

Abnormal Development (Carcinogenesis)

In the normal physiological evolution of the protovirus DNA, information from the environment would be effective only as it affected the general state and differentiation of the organism. However, protovirus evolution could by some random events give rise to a virus which could enter an organism from the outside. Because the virus has the ability to insert information into the cell, it could short-circuit the requirement for protovirus evolution. For example, once RNA sarcoma viruses were formed, they would upon infection make new DNA which would contain all of the information necessary to bring about neoplastic transformation. No multiple cycles of DNA→RNA→DNA information transfer need occur

However, apart from laboratory conditions, such single-step transfer processes might never lead to cancer.

In normal development, this DNA→RNA→DNA information transfer could be used to identify cells as being of a particular type and to recruit other cells into a related or identical form. A cell's particular differentiated state could be partially specified by the types of new protovirus-derived DNA it contained. RNA transcribed from this new protovirus-derived DNA could be transferred to other cells and thus induce an orderly conversion of the other cells into the same or a related differentiated state. Examples of such differentiation might be the secondary antibody response (*10*) and embryonic induction.

The usual process leading to cancer could be a variation in the normal physiological evolution of the protovirus DNA, so that variants which contain information for the cancer appeared either by mutation of the base sequences or by integration in incorrect places or both. Since DNA→RNA→DNA information transfer involves DNA, RNA, and enzymes (protein), the formation of variants may result from the response of any of these molecules to irradiation or chemicals. This oncogenic process would not require the formation of an RNA tumor virus. It would require only alteration of the information in cells, so that there was the information which was necessary for cancer. This information for cancer may or may not be homologous to the information in RNA tumor viruses. The nature of this information is not considered here (*see 2*, *11*).

In extreme cases, one could imagine that a product of protovirus evolution would infect the germ line, become integrated there, and thus also affect progeny organisms. Such a process could provide part of a mechanism for inheritance of some acquired characters. Selection at the level of the germ cells and at the level of the organism would also be involved.

This protovirus theory was derived *a priori* from consideration of the origin of RNA sarcoma viruses. It also explained the presence in uninfected cells of DNA homologous to a portion of the RNA of RNA tumor viruses (*12*, *13*), and the apparent vertical transmission of the information for RNA tumor viruses (*1*, *14*).

Since the protovirus theory was derived, several new experimental findings have been made which are relevant to it. An RNA-directed DNA polymerase has been found in virions of visna virus and simian foamy virus (*15*, *16*). Neither of these viruses has so far been associated with neoplasia. Preliminary evidence suggests that RNA-directed DNA polymerase may also be involved in gene amplification in oocytes of Xenopus (*5*). Uninfected chicken cells may contain an element which is inherited as a single Mendelian dominant gene and can become infectious after phenotypic mixing with an RNA tumor virus (*17*). This element could be a protovirus-derived element that was not yet a complete virus genome. It also could be a defective provirus. Antigens related to RNA tumor viruses are found in normal mouse embryos (*18*). These antigens could represent the RNA-directed DNA polymerase and other proteins of the protoviruses.

Finding an RNA-directed DNA polymerase in uninfected tissues of normal organisms may be the first direct evidence bearing on this hypothesis. Knowledge of the specificities and time of occurrence of such a polymerase would aim in demonstrating the type of information transfer postulated here.

However, even if this protovirus theory is correct in its essentials, it raises the question of how carcinogens cause the misevolution of protoviruses to give rise to information for the neoplastic phenotype and how such information in protovirus-derived DNA actually causes the neoplastic phenotype.

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ADDENDUM

This Editorial was one of the first of a number of articles in which I described the protovirus hypothesis. Since then, our knowledge of RNA tumor viruses or retroviruses, as they are now called, and the relationships of retroviruses to cancer and to normal cells has increased enormously. The major features of the protovirus hypothesis described in the Editorial, i.e., the existence of DNA to RNA to DNA information transfer in normal organisms and carcinogenesis as a result of misevolution of this process, have only been confirmed for some vertically transmitted retroviruses, especially AKR murine leukemia virus and the precursors of radiation murine leukemia virus. In addition, reverse transcriptases have been found in some normal avian cells, and virus-related proteins and nucleic acids are widespread in many other normal cells.

Retroviruses can be described as strongly transforming (rapidly oncogenic), weakly transforming (slowly oncogenic), or not oncogenic. The various protovirus hypotheses can be stated now as follows:

1) Strongly transforming retroviruses evolve from weakly transforming retroviruses and cellular DNA.

2) Weakly transforming retroviruses cause neoplasia by forming strongly transforming genes.

3) Precursors of retroviruses are present in many cells.

4) Weakly transforming retroviruses evolve from cellular DNA.

5) Nonviral cancers are the result of the formation of strongly transforming genes by processes related to those involved in the evolution of retroviruses.

6) Cellular retrovirus-related precursors and processes play a role in normal cellular development.

These hypotheses are supported to different extents by data presently available. Hypotheses 1–4 have more supportive data than does hypothesis 5; no data directly substantiate hypothesis 6.

An alternate hypothesis to explain some of these data is the germlike persistence and gradual mutation of originally horizontally transmitted retroviruses.

The hypothesis of the presence of the genome of a strongly transforming retrovirus in the genome of all cells and its activation during carcinogenesis (oncogene hypothesis) is not, however, supported by data now available.

The rapid pace of experimentation in this field will undoubtedly test these hypotheses further.

GUEST EDITORIAL——————————————**by Henry Harris, F.R.S.**

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The Croonian Lecture, 1971

Cell Fusion and the Analysis of Malignancy [1]

THE CELLS of the body do not normally engage in sex. Nor is it easy to see that sexual activity would greatly benefit them. For sex is ultimately merely a device to facilitate the accumulation in a single individual of favourable mutations occurring separately in different individuals; and since the cells in the body are, at least in large part, genetically identical, the advantages to be gained by genetic exchange are obviously limited. In recent years, however, a technique has been devised that imposes a form of artificial sexuality on somatic cells, and it has been found that somatic cells of widely different genetic constitutions can be induced to undergo genetic amalgamation and exchange. A few years ago, Professor Hayes, in a Leeuwenhoek Lecture (Hayes 1966) described how sex in bacteria is mediated by an infectious particle which produces a change in the cell wall of the 'male' bacterium that enables it to make intimate contact with the 'female' bacterium. A connexion is then established between the cytoplasms of the two bacteria and through this connexion transfer of genetic material may take place. The imposition of sexuality on somatic cells is achieved by a mechanism which, viewed superficially, is reminiscent of bacterial conjugation. An animal virus, whose normal mode of entry into the cell appears to involve fusion between the viral membrane and the cell membrane, is used to facilitate fusion between the cell membranes of contiguous cells (Okada 1958; Harris & Watkins 1965). Cytoplasmic bridges are thus established which eventually determine the complete coalescence of the cytoplasms of adjacent cells (Schneeberger & Harris 1966). In this way multinucleated cells are formed which contain various numbers of nuclei, and different kinds of nuclei if cells of different kinds are brought together (Harris, Watkins, Ford & Schoefl 1966). The virus now commonly used to produce cell fusion is the Sendai virus, a member of the parainfluenza group of myxoviruses, although many other viruses can achieve the same effect. Unlike the sex particle in bacteria, however, Sendai virus will produce fusion of somatic cells even after its nucleic acid has been destroyed (Okada & Tadokoro 1962; Neff & Enders 1968); the viral envelope is all that is required for this effect. The standard reagent for inducing cell fusion is Sendai virus inactivated by large doses of ultraviolet light or by appropriate treatment with β-propriolactone.

Most cell types from a very wide range of animal species are susceptible to the action of Sendai virus, so that fusion can be achieved between cells from different animal species and cells showing very different forms of specialization. I have already reviewed before this Society the general properties of such fused cells (Harris 1966). These cells are, as I have said, initially multinucleate; and many experiments have now been done on the interactions of different nuclei and cytoplasms brought together in this way (Harris 1970). But for the purposes of the present lecture I shall be concerned with the mononucleate hybrid cells that are produced when the nuclei of a binucleate (and occasionally trinucleate) cell fuse together. This nuclear fusion takes place when the binucleate cell enters mitosis. The two nuclei commonly enter mitosis together; and, when this occurs, a single spindle may be formed and all the chromosomes may become alined along one metaphase plate. The cell then divides to give rise to two mononucleate daughter cells each of which contains within a single nucleus the chromosomes of both parent cells. Many of these composite mononucleate cells, even when they are derived from parents of widely different animal species, are capable of indefinite multiplication.

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Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

Genetic analysis of somatic cells requires, however, not only that different chromosomal complements be brought together; the chromosomes must also segregate. Hybrid somatic cells do not appear to undergo the regular systematic segregation normally seen at meiosis; but for the purposes of genetic analysis a useful alternative is provided by chromosome loss (Ephrussi, Scaletta, Stenchever & Yoshida 1964). The mechanisms responsible for loss of chromosomes in hybrid cells remain rather unclear, despite a good deal of intensive study. Virtually all such cells undergo a slow progressive loss of chromosomes on continued cultivation, and this may be determined in some cases by non-disjunction (Handmaker 1971). In certain cell combinations large numbers of chromosomes are eliminated during the early cell divisions, possibly at the very first division (Weiss & Green 1967; Nabholz, Miggiano & Bodmer 1969). Some evidence suggests that these drastic early losses may be produced by spindle abnormalities of one kind or another. Whatever the mechanism of chromosome loss, the end result is the production of cells which, from the genetic point of view, may be regarded as segregants of the original hybrid cell population.

The experiments I now wish to describe had a very simple objective: to determine whether the daughter cells resulting from the fusion of a malignant cell and a non-malignant one were malignant or not. (Malignancy is here defined as the ability of tumour cells to grow progressively and kill their host.) One might at first glance suppose that the solution of so simple a problem would not pose any great difficulty; but when one considers that the injection of cells into an animal selects for malignancy and that hybrid cells continually generate segregants from which certain parental chromosomes are eliminated, the complexities of the investigation become apparent. The work began as a collaboration between Professor George Klein of the Department of Tumour Biology at the Karolinska Institute and myself. At various stages we were joined by Dr O. J. Miller and Miss D. Quinn at Oxford and Drs P. Worst, T. Tachibana, Y. Bregula and F. Weiner at Stockholm. The enterprise would long ago have foundered but for the skill and enthusiasm of these colleagues.

If we were to have any hope of obtaining an unequivocal answer to our question it was clear that, to begin with, we should have to use tumour cells whose malignancy was not in doubt. We therefore chose for our experiments six transplantable mouse tumours, which grew as well in the solid as in the ascitic form, and which produced progressive fatal neoplasms in 100% of genetically compatible mice with inocula of 100 cells or less. More lethal cell populations can hardly be obtained. Table 1 lists the six tumours and some of their properties. The Ehrlich tumour appears originally to have been a spontaneous mammary carcinoma. It arose in Ehrlich's laboratory in a mouse of unknown genetic constitution and has been passaged continuously in many different strains of mice for more than seventy years. The cells of this tumour possess remarkably efficient mechanisms for masking histocompatibility antigens and hence grow freely in any strain of mouse. SEWA and MSWBS are both sarcomas, the first induced by polyoma virus, the second by the chemical carcinogen methylcholanthrene. Both tumours bear the H-2^s group of histocompatibility antigens and their growth is limited to mice of the A.SW strain. SEWA also bears the specific transplantation antigen characteristic of polyoma virus infection.

TABLE 1.—Experimental tumours

Tumour	Type	Origin	Genotype of host	Histocompatibility class
Ehrlich	Mammary carcinoma	Spontaneous	?	?
SEWA	Sarcoma (osteogenic)	Polyoma virus	A.SW	H-2^{s}
MSWBS	Sarcoma	Methylcholanthrene	A.SW	H-2^{s}
YAC	Lymphoma	Moloney virus	A	H-2^{a}
YACIR	Lymphoma	Moloney virus	A	H-2^{a}
TA3	Mammary carcinoma	Spontaneous	A	H-2^{a}

YAC is a lymphoma induced by Moloney virus. It bears the H-2^{a} group of histocompatibility antigens and a specific surface antigen characteristic of Moloney virus infection. The tumour grows only in A strain mice. YACIR is a subline of YAC selected for diminished expression of the Moloney virus-induced surface antigen by continuous passage in mice immunized against this antigen. TA3 is a spontaneous mammary carcinoma which arose originally in an A strain mouse in T. S. Hauschka's laboratory. It bears the H-2^{a} group of histocompatibility antigens but readily transgresses histocompatibility barriers.

For technical reasons these tumours were first fused with two derivatives of the L cell line, an established line of mouse fibroblasts that has been maintained in culture for more than fifteen years. One of these derivatives, the A9 cell, was selected for resistance to 8-azaguanine and lacks the enzyme inosinic acid pyrophosphorylase; the other, the B82 cell, was selected for resistance to 5-bromodeoxyuridine and lacks the enzyme thymidine kinase (Littlefield 1964). These enzymic defects in the L cell derivatives facilitate the selection of the hybrid cells, for culture media can be employed that inhibit the growth of cells lacking these enzymes. However, since it was possible that the growth of the hybrid cells *in vivo* might be influenced by the presence of a severe enzymic defect in one of the parent cells, hybrids were also constructed between some of the tumour cells and a presumptive revertant of the A9 cell in which inosinic acid pyrophosphorylase activity had been restored. This cell line, known as A9RI, arose spontaneously in cultures of A9 cells maintained in a selective medium that inhibits the growth of cells lacking inosinic acid pyrophosphorylase. In the case of crosses between the A9RI cells and the tumour cells, a selective medium could not be used to facilitate the isolation of the hybrid cells; and these were obtained by procedures that exploited differences in surface properties between the hybrids and the parental cell lines. Differences in surface properties formed the basis of successful selective procedures for several other hybrid lines that could not be isolated by means of selective media. Where the cultural characteristics of the hybrids closely resembled those of the parental cells, and no selective medium was appropriate, the fused cell population was cloned, and clones having morphological features suggesting hybridity were isolated. Karyological investigation of these clonal populations eventually permitted the identification, and hence isolation, of the desired hybrids.

The L cell line was originally derived from C3H mice and thus bore the H-2^{k} histocompatibility antigen complex. This complex was also present on the surface of the three L cell derivatives, A9, B82 and A9RI. As shown in table 2, these three cell lines had a very low level of tumorigenicity in syngeneic C3H mice: even when assayed in X-irradiated (4 J kg^{-1}) newborn mice, which provide optimal conditions for cell growth *in vivo*, these cells produced tumours in only 6 to 33% of the recipients with inocula between 5×10^4 and 2×10^6 cells. The 50% tumour dose (the inoculum required to produce tumours in 50% of the recipient animals) exceeded that of the tumour cells by a factor of at least 10^5.

Hybrid cells are usually identified by their karyotype: they contain in varying proportions the chromosomes of both parent cells. In table 3 the

TABLE 2.—Growth of L cell derivatives *in vivo*

Cell type	No. takes (total No. animals with progressive tumours/total No. inoculated)	Percentage takes
A9	3/25	12
B82	3/48	6
A9RI	22/66	33

All animals were X-irradiated newborn C3H mice. Inocula varied between 5 × 10^4 and 2 × 10^6 cells injected subcutaneously.

chromosomal constitutions of the parental cells are shown, and in table 4 the chromosomal constitutions of the hybrid cells derived from them. It will be seen that, in general, the hybrid cells initially have chromosome numbers approximating to the sum of the two parental chromosome sets. In some cases the modal chromosome number of the hybrid cells is exactly what one would expect from the fusion of two modal parent cells; in other cases the hybrid cells have a slightly lower chromosome number than that to be expected from the sum of the two parental modes. The hybrid nature of the cells was confirmed in all cases by the presence of parental marker chromosomes, or by the presence of the two parental H-2 antigen complexes, or both. As shown in table 4, the hybrid cells lose chromosomes on progressive cultivation *in vitro*; but the loss is very gradual and little change in chromosome number is usually seen in the first few weeks. These hybrids between the tumour cells and the established cell lines thus resemble many other intraspecific hybrid cells that have been described in having relatively stable chromosomal constitutions initially composed of something pretty close to the sum of the two parental chromosomes sets (Ephrussi *et al.* 1964; Littlefield 1966; Engel, McGee & Harris 1969).

TABLE 3.—Chromosomal constitution of parental cell lines

Cell line	Total chromosome No.		No. bi-armed chromosomes	
	Range	Mode	Range	Mode
In vitro				
A9	50–69	56	22–27	25
B82	53–70	57	23–29	27
A9RI	37–52	49	14–26	23
Tumour				
Ehrlich	71–94	76	0–2	1
SEWA	42–44	43	0–1	1
MSWBS	28–30	29	8–11	10
YAC	39–41	40	0–1	1
YACIR	40–42	40	0	0
TA3	40–41	40	0	0

In testing hybrid cells for their ability to grow progressively *in vivo* it is, of course, obvious that histocompatible test animals must be used. This means, in practice, that where the two parent cells are derived from mice of known genotypes the hybrid cells are tested in the F_1 progeny of the parental mouse strains. All the tumour cell lines grew as well in the F_1 hybrid animals as in the parental strains. However, it was possible that prolonged cultivation *in vitro* might have generated minor degrees of histoincompatibility between the cell lines and the animals from which they were initially derived; and it was also possible that new antigenic combinations might have been generated by the cell fusion itself. For this reason our tests for malignancy were carried out on genetically

TABLE 4.—Chromosomal constitution of hybrid cell lines

Cell line	Date of examination	Total chromosome No.		No. bi-armed chromosomes	
		Range	Mode	Range	Mode
Ehrlich/A9	15. x. 68	104–150	128	11–30	24
Ehrlich/A9	12. ix. 69	71–87	83	11–26	16
Ehrlich/A9	25. v. 70	65–108	(73–77)	13–23	16
SEWA/A9	10. ii. 69	81–113	94	17–29	21
SEWA/A9	27. x. 69	71–95	(84–87)	20–30	23
MSWBS/A9	3. iii. 69	65–109	86	29–40	36
MSWBS/A9	5. ix. 69	72–85	(80–82)	27–38	32
Ehrlich/B82	9. ix. 69	86–156	112	13–30	(22)
Ehrlich/A9RI	26. vi. 69	74–136	(118–122)	12–26	(20)
Ehrlich/A9RI	5. ix. 69	85–105	(96–98)	17–24	20
YAC/A9	23. iii. 70	83–103	88	24–28	26
YACIR/A9	2. iv. 70	78–94	92	26–28	27

Parentheses denote weak mode.

compatible newborn animals that had received 4 J kg^{-1} of whole body irradiation before test. Since these X-irradiated newborn mice have little immunological reactivity and permit the growth of tumours bearing foreign histocompatibility antigens, we felt confident that our tests would not be complicated in any important way by histoincompatibility reactions between cells and host. I shall present evidence later that our confidence was not misplaced.

The first hybrid cells we tested were those resulting from the fusion of Ehrlich cells with A9 cells; and it was at once obvious that the tumorigenicity of these hybrids was in no way comparable to that of the Ehrlich cells. With inocula between 3×10^4 and 3.5×10^6 cells, tumours developed in only about a third of the X-irradiated newborn mice. This take incidence was of the same order as that obtained with A9 cells. In statistical terms, these results suggested that in the hybrid cell population only one cell in about 10^5 or 10^6 was able to produce a tumour, whereas virtually every Ehrlich cell can do so. The question, of course, at once arose whether the very small minority of hybrid cells that did give rise to tumours differed in their chromosomal constitution from the hybrid cell population as a whole. Karyotypes of the cells in the tumours arising from Ehrlich/A9 hybrids were therefore examined. It was found that in all cases the tumours had chromosome numbers much lower than that of the hybrid cells injected. Whereas the modal chromosome number of the latter was about 128, the tumour cell populations had modes in the region of 80. The tumours were none the less derived from hybrid cells, for their karyotypes contained marker chromosomes from both parent cells. These results thus indicated that the hybrid cells with chromosome complements approximating to the sum of the two parental chromosome sets had very little, if any, ability to grow progressively *in vivo;* the tumours were all composed of cells from which certain chromosomes had been eliminated.

This result raised several obvious questions. (1) Is the suppression of malignancy by cell fusion limited to the Ehrlich ascites tumour? (2) Is the effect peculiar to the A9 cell? (3) Does the metabolic defect in the A9 cell (inosinic acid pyrophosphorylase deficiency) play an important role in the suppression of malignancy? (4) Can the suppressive effect be exercised by normal diploid cells? (5) Is it in general the case that when a malignant cell and a non-malignant cell are fused together, the hybrids with unreduced chromosome complements are not malignant? (6) Is a loss of chromosomes essential for the production of malignant variants from a non-malignant hybrid cell population? (7) If loss of chromosomes is essential, is it necessary to eliminate certain specific chromosomes, or is it enough simply to achieve some overall reduction in chromosome number?

Tests on SEWA/A9, MSWBS/A9, YAC/A9 and YACIR/A9 hybrids at once revealed that the suppressive effect was not limited to the Ehrlich cell; the malignancy of all these tumours was dramatically reduced by fusion with the A9 cell. As shown in table 5, the take incidences for all these hybrids were not very different from those obtained with A9 cells. Tests on Ehrlich/B82 and Ehrlich/A9RI hybrids gave similar results. It was thus not only the A9 cell that had the ability to suppress malignancy, and not only L cell derivatives with hereditary metabolic defects. The A9RI cells did not have the metabolic defect of A9 cells (inosinic acid pyrophosphorylase deficiency), but the take incidence for the Ehrlich/A9RI hybrids, although a little higher than that obtained with the Ehrlich/A9 hybrids, was actually lower than that obtained with the A9RI cells themselves. The 50% tumour doses for all these hybrids were about 10,000 times higher than those for the parental tumours. Examination of the chromosomal constitutions of the tumours arising from the injection of the hybrid cells revealed in all cases a situation essentially similar to that found with the Ehrlich/A9 hybrids. The tumours were not produced by progressive growth of the hybrid cells injected but by selective overgrowth of a minority cell population with a lower modal chromosome number.

Several tumours derived from Ehrlich/A9, Ehrlich/B82 and Ehrlich/A9RI hybrids were explanted, grown continuously *in vitro* for 2 months, and then tested again for growth *in vivo*. As shown in table 6, 100% take incidences were obtained in all cases. This result confirms the karyological observations in demonstrating that the hybrid tumours were indeed produced by cell selection; and it further shows that continued cultivation of

Table 5.—Growth of hybrid cells *in vivo*

Cell type	Genotype of host	No. takes (total No. animals with progressive tumours/total No. inoculated)	Percentage takes
Ehrlich/A9 (Jan. to June 1969)	C3H	4/12	33
Ehrlich/A9 (July to Dec. 1969)	C3H	2/32	6
Ehrlich/A9 (Dec. 1969 to June 1970)	C3H	2/38	5
SEWA/A9	C3H × A.SW F1	8/25	32
MSWBS/A9	C3H × A.SW F1	13/30	43
Ehrlich/B82	C3H	3/41	7
Ehrlich/A9RI	C3H	25/96	26
YAC/A9	C3H × A F1	1/27	4
YACIR/A9	C3H × A F1	6/52	12

Inocula varied between 3×10^4 and 3.5×10^6 cells.

the cells *in vitro* does not in itself suppress malignancy. It thus appeared that when any of these highly malignant tumour cells were fused with L cell derivatives of low tumorigenicity, the latter contributed to the hybrid cell some factor or factors that suppressed the malignant phenotype of the tumour cell. The hybrid cells resulting from such fusions had little or no ability to grow progressively in the animal so long as they retained the complete chromosome sets of both parent cells; but when certain chromosomes were eliminated, the malignant phenotype reappeared and the segregant cells were again able to produce tumours.

The question remains whether restoration of the malignant phenotype requires the elimination of certain specific chromosomes or whether a nonspecific reduction in chromosome number will suffice. An analysis of the tumorigenicity of Ehrlich/A9 hybrids after prolonged cultivation *in vitro* provides an answer to this question. Although chromosome loss in this hybrid takes place very slowly *in vitro*, after a period of eighteen months' cultivation the modal chromosome number of the hybrid cell population was reduced from an initial 128 to about 80. This reduction was thus comparable to that seen in the tumours arising from these hybrids where modal chromosome numbers in the region of 80 were usually found. But, as shown in table 5, the take incidences for these hybrids with chromosome numbers reduced by prolonged cultivation *in vitro* were, if anything, even lower than those obtained with the original unreduced hybrid cell population. It is therefore clear that to generate malignant segregants from the non-malignant hybrid cells, it is not enough simply to reduce the overall number of chromosomes in the cell; certain specific chromosomes have to be eliminated. These results also imply that injection of the hybrid cells into the animal must select for the loss of a different set of chromosomes from those eliminated by growth *in vitro*. Analysis of the patterns of elimination of parental marker chromosomes in the two situations has confirmed that this is indeed the case.

Table 6.—Growth of hybrid tumour cells *in vivo* after 2 months' cultivation *in vitro*

Cell type	No. takes (total No. animals with progressive tumours*/total No. inoculated)
Ehrlich/A9 (1)	4/4
Ehrlich/A9 (2)	4/4
Ehrlich/A9 (3)	4/4
Ehrlich/B82	8/8
Ehrlich/A9RI	4/4

*All animals were dead within 3 to 4 weeks of inoculation.

Can a normal diploid cell suppress the malignant phenotype? Hybrids were made between Ehrlich cells and diploid fibroblasts isolated directly from CBA mice homozygous for the T6 chromosome translocation, which provides an easily identifiable marker for the diploid fibroblast component in the hybrid cell. Three wild type hybrid populations were tested first, each derived from a separate fusion experiment. With inocula comparable to those used in testing the Ehrlich/A9 hybrids, take incidences close to 100% were obtained for all three populations. But wild type populations are populations grown under conditions that select for the most rapidly growing cells. It therefore seemed worth while to examine a number of clonal populations, each derived from a separate primary fusion, for it was possible that the tumorigenicity of some of the clones might differ from that of the wild type populations. The take incidences for a number of clonal populations of Ehrlich/fibroblast hybrids are shown in table 7. Although all of these populations gave high take incidences, it will be seen that some of the clones, even with inocula up to 2 × 10^6 cells, did not give the 100% take incidence that would be expected for Ehrlich cells; and measurement of the growth rate of some of the tumours produced from these hybrids also showed some variation from clone to clone. It thus appeared that fusion with diploid fibroblasts had produced some changes in the capacity of the Ehrlich cells to grow progressively *in vivo*, but these changes were not very impressive.

One might be tempted to conclude that diploid fibroblasts were essentially ineffective, or at least much less effective than the L cell derivatives, in suppressing the malignancy of the Ehrlich tumour cell. But analysis of the chromosomal constitutions of the Ehrlich/fibroblast hybrids again revealed a situation of greater complexity. The expected chromosome number for a hybrid cell produced by the fusion of one modal Ehrlich cell and one diploid fibroblast would be 116. However, as shown in figure 1, all populations of Ehrlich/-fibroblast hybrids, except some grossly polyploid clones, showed modal chromosome numbers between 91 and 102. Such reduced modal chromosome numbers were seen as soon as enough cells could be generated to permit chromosome analysis, and it has, if fact, not proved possible to isolate a population of Ehrlich/fibroblast hybrids with a chromosomal complement corresponding to the sum of the two parental modes. Chromosome losses apparently occur very rapidly in these hybrids, and karyological analyses of populations grown continuously *in vitro* indicate a high degree of chromosomal instability compared with the hybrids made by fusing the Ehrlich cells with L cell derivatives. It is possible that this instability may be related to the fact that the L cell derivatives have long been adapted to growth *in vitro*, whereas the freshly isolated fibroblasts have not; but, whatever the cause of the instability, it precludes any firm conclusion about the capacity of diploid fibroblasts to suppress malignancy. For rapid and extensive elimination of chromosomes will obviously greatly increase the chances of producing malignant segregants in the hybrid cell population and will thus tend to generate high take incidences. The tumours produced from Ehrlich/-fibroblast hybrids showed substantial variation in chromosome number; but no tumour was found with a chromosomal constitution corresponding to that expected for the complete sum of the two parental chromosome sets.

TABLE 7.—Growth of Ehrlich/fibroblast hybrids *in vivo*

Cell line or clone	No. takes (total No. animals with progressive tumours/total No. inoculated)	Percentage takes
Ehrlich/T6T6 (wild type)	123/123	100
Clonal series		
Clone 1	12/12	100
Clone 2	27/28	97
Clone 3	20/20	100
Clone 4	36/45	80
Clone 5	11/11	100
Clone 7	7/8	88
Clone 7*b*	11/12	92
Clone 8	26/28	93
Clone 9	10/13	77
Clone 11	6/10	60

Inocula varied between 10^4 and 2 × 10^6 cells.

The Ehrlich tumour is highly aneuploid and its cells have a wide range of chromosome numbers. It seemed possible that if the diploid fibroblasts were fused with tumour cells showing a less variable chromosome number, some hybrid lines might be produced containing essentially complete parental chromosome sets. It was reasonable to hope that such hybrid lines, if they could be obtained, might provide a less equivocal test of the ability of the diploid cell to suppress malignancy. The fibroblasts were therefore fused with SEWA cells, which show a narrow range of 42 to 44 chromosomes (with a mode of 43), and with TA3 cells, which are virtually euploid. For the experiments with the SEWA cells, CBA fibroblasts homozygous for the T6 translocation were again used; but, in order to

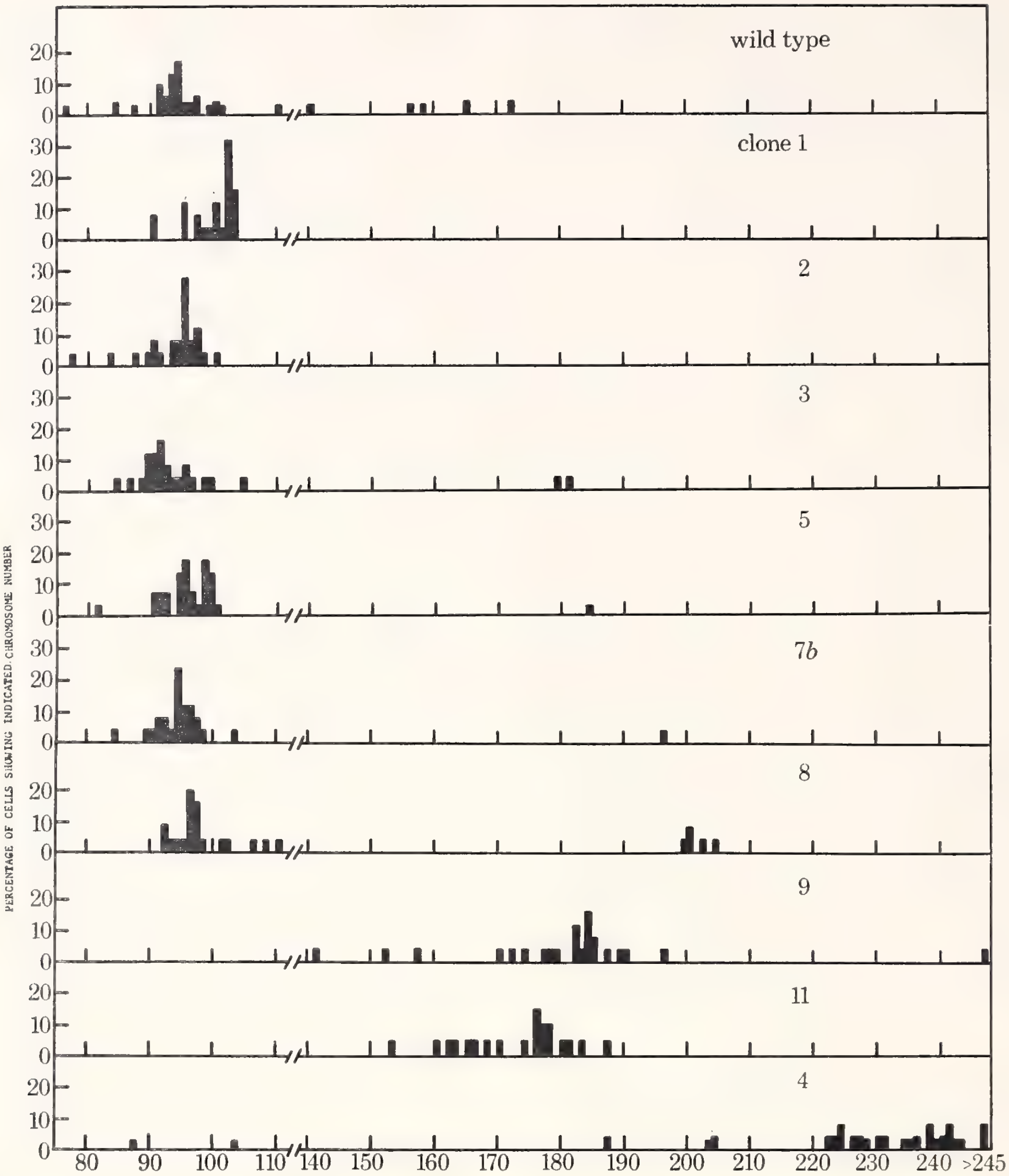

Figure 1. Distribution of chromosomes in clonal populations of Ehrlich/fibroblast hybrids. The expected chromosome number for a hybrid cell resulting from the fusion of one modal Ehrlich cell and one diploid fibroblast would be 116. Except for grossly polyploid clones, all the hybrid populations have chromosome numbers well below that to be expected for the sum of the two parental chromosome sets.

eliminate the possibility that some complication might be introduced by the presence of this translocation, the TA3 cells were fused with karyologically normal fibroblasts isolated from ACA mice. (ACA mice have a private histocompatibility system which permits ready identification of the hybrid cells by immunological methods.) Figure 2 shows the chromosomal constitutions of fifteen clones of SEWA/fibroblast hybrids, each derived from a different primary fusion. The expected chromosome number for a hybrid cell containing one modal chromosome set from the SEWA cell and one diploid chromosome set would be 83; but it will be seen that, as soon as they could be analysed, the majority of the clonal populations already had chromosome numbers a good deal short of this. However, five clones did initially show modes in the region of 80 and some of these retained this high chromosome number for several weeks of subcultivation *in vitro*, long enough, in any case, to permit the tumorigenicity of the clones to be tested. The results of the *in vivo* tests are shown in table 8. It will be seen that all the clones with reduced chromosome numbers gave take incidences not far short of 100%; but in three of the clones with initially unreduced chromosome numbers the take incidences were greatly reduced. Compared with the parental SEWA tumour cells, these reduced take incidences represent increases in the 50% tumour doses of at least 10^5. It thus appears that the diploid fibroblast can suppress the malignant phenotype, but only in hybrid cells in which something close to the complete chromosome sets of both parent cells is retained. This conclusion is supported by chromosomal analysis of some forty tumours produced by the injection of the SEWA/fibroblast hybrid cells. The cells in these tumours showed a large spread of chromosome numbers, as might be expected from the chromosomal instability of the hybrid cells *in vitro;* but, even so, the modes, where clear modes could be established, indicated that the tumours were dominated by cells from which substantial numbers of chromosomes had been eliminated. No tumour was found composed of cells with chromosomal constitutions corresponding to the complete sum of the two parental chromosome sets.

TABLE 8.—Growth of SEWA fibroblast hybrids *in vivo*

Cell line	No. takes (total No. animals with progressive tumours/total No. inoculated)	Percentage takes
SEWA/T6T6 (wild type)	15/16	94
Clonal series		
Clone 1	4/4	100
Clone 2	11/19	58
Clone 3	13/27	48
Clone 4	7/7	100
Clone 5	6/6	100
Clone 6	3/3	100
Clone 7	7/7	100
Clone 10	12/12	100
Clone 13	16/31	51
Clone 14	15/15	100
Clone 15	3/3	100
Clone 16	18/20	90

Inocula varied between 1.2×10^4 and 3.6×10^6 cells.

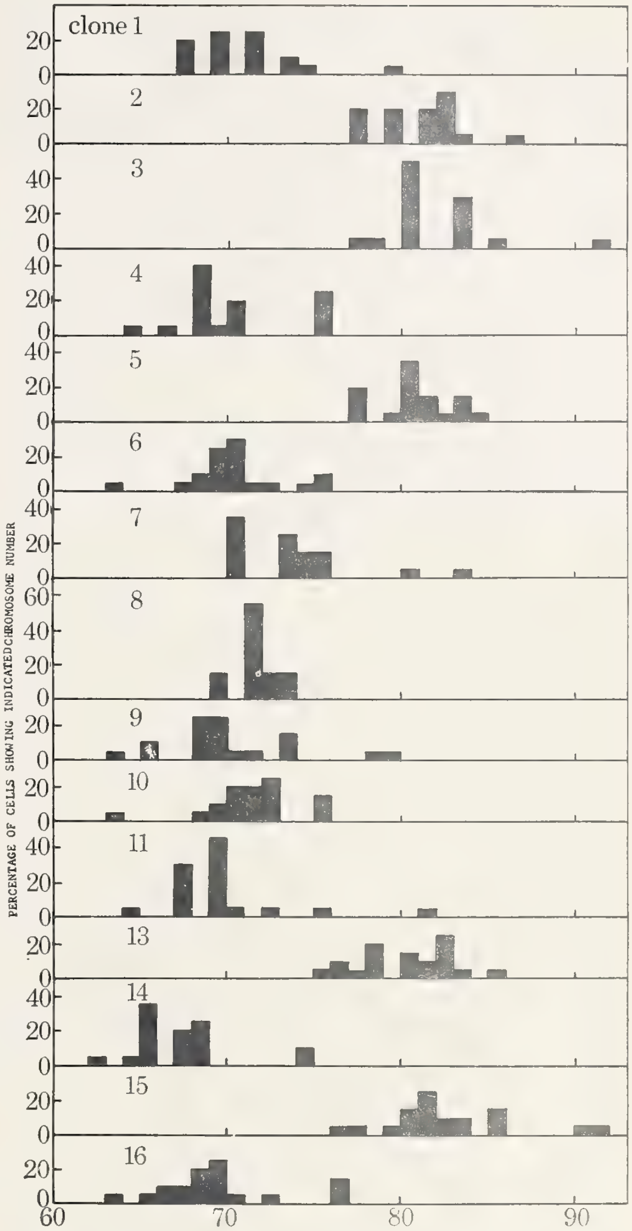

FIGURE 2. Distribution of chromosomes in clonal populations of SEWA/fibroblast hybrids. The expected chromosome number for a hybrid cell resulting from the fusion of one modal SEWA cell and one diploid fibroblast would be 83. Most of the clones have chromosome numbers well below that to be expected for the sum of the two parental chromosome sets, but clones 2, 3, 5, 13 and 15 have modes close to this figure.

Since our results showed that the tumorigenicity of Ehrlich/fibroblast and SEWA/fibroblast hybrids could be profoundly influenced by chromosome loss, a special study was made of TA3/ACA fibroblast hybrids that were selected for high chromosome numbers. Populations of cells were isolated with chromosome numbers exceeding the sum of the two parental chromosome sets (80). These cells, with modes between 110 and 120 must at one stage have contained at least three parental chromosome sets. It was hoped that such hybrids might retain the chromosomes responsible for suppression of malignancy over a longer period of cultivation and thus provide a more decisive demonstration of this suppression. Table 9 shows the results of the *in vivo* tests on three such populations, one wild type and two clonal. It will be seen that the take incidences, despite the high inocula, are very low, comparable, in fact, to those obtained with hybrids between tumour cells and L cell derivatives. Chromosomal analysis of the tumours arising from the TA3/fibroblast hybrids showed once again that the hybrid tumours were not produced by the progressive growth of the whole cell population injected, but by the selective overgrowth of cells from which chromosomes had been eliminated. Whereas the modes of the three cell populations injected were 117, 112 and 114, no tumour had a mode greater than 103, and the great majority had modes in the region between 80 and 90. These findings thus indicate that the differences in overall take incidence between the hybrids produced by fusing the tumour cells with L cell derivatives and those produced by fusing the tumour cells with diploid fibroblasts are not to be explained by the greater capacity of the L cell derivatives to suppress malignancy. The higher take incidences seen with tumour/fibroblast hybrids are due to their chromosomal instability which generates malignant segregants at a much higher frequency. That this is the correct interpretation of the data is supported by an examination of the take incidences of tumour/fibroblast hybrids as a function of the length of time that the cells have been grown *in vitro*. In table 10 the take incidences of individual batches of TA3/fibroblast hybrids are set out in a chronological order that reflects the period of cultivation *in vitro*. It will be seen that these hybrids initially have virtually no capacity for progressive growth *in vivo;* but, as the period of

TABLE 9.—Growth of TA3/fibroblast hybrids *in vivo*

Cell line	Chromosome No.		No. takes (total No. animals with progressive tumours/total No. inoculated)	Percentage takes
	Mode	Range		
Wild type	117	100–117	19/54	35
Clone 5	112	110–121	6/19	32
Clone 7	114	66–121	2/10	20

Inocula varied between 1.2×10^4 and 3.6×10^6 cells.

TABLE 10.—Progressive increase in take incidence of TA3/fibroblast hybrids on continued passage *in vitro*

Cell type	Date of inoculation	No. cells inoculated	Final date of examination	Genotype of host	No. takes
TA3/fibroblast hybrid (clone 6)	11. vi. 70	4.1×10^4	24. vii. 70	A	0/3
	11. vi. 70	4.1×10^4	10. ix. 70	A	0/3
	2. vii. 70	4.4×10^4	18. ix. 70	A × ACA	0/1
				A	0/1
	5. vii. 70	1.6×10^4	3. ix. 70	A	0/17
	20. vii. 70	1.6×10^4	12. x. 70	A × ACA	0/1
	4. viii. 70	3.2×10^4	23. x. 70	A	1/4
	10. viii. 70	2.4×10^4	23. x. 70	A	0/2
	13. viii. 70	5.7×10^4	3. xii. 70	A	1/1
	20. viii. 70	3.3×10^4	18. ix. 70	A × ACA	1/1
	20. viii. 70	3.3×10^4	25. ix. 70	A × ACA	1/1
	24. viii. 70	8.2×10^4	23. x. 70	A × ACA	2/3
				A	3/3
	17. ix. 70	6.3×10^4	3. xii. 70	A	1/4
	28. ix. 70	1.8×10^4	23. x. 70	A	3/3
	22. iii. 71	9.0×10^4	19. v. 71	A × ACA	2/3

cultivation *in vitro* is extended and chromosome losses supervene, a marked increase in the take incidence occurs.

In using X-irradiated newborn syngeneic mice for our assays of growth *in vivo*, we chose, from the immunological point of view, the least reactive system available. If these animals do not provide immunologically neutral conditions for testing the tumorigenicity of the hybrid cells, it is difficult to see how this property can be tested at all. I now wish to present the evidence for the conclusion that these animals did provide a satisfactory test system and that our assays for tumorigenicity were not complicated to any important degree by histoincompatibility between the inoculum and the host. As I have mentioned previously, these X-irradiated newborn mice permit the growth of tumours bearing foreign histocompatibility antigens; and it was therefore not surprising that where populations of hybrid cells showed high or low tumorigenicity when tested in X-irradiated newborn syngeneic mice, these differences in tumorigenicity were also apparent when the hybrid cells were tested in X-irradiated newborn allogeneic mice. This was the case even for different clonal populations of the one hybrid cell type. Different clones of SEWA/fibroblast hybrids showed marked differences in tumorigenicity when assayed in X-irradiated newborn A.SW × CBA F_1 hybrid mice (syngeneic); but the differences in tumorigenicity between these clones could also be revealed by assays in X-irradiated newborn A.SW or CBA mice (allogeneic). Table 10 shows that the rising take incidence of TA3/fibroblast hybrids on continued cultivation *in vitro* could be demonstrated not only in the syngeneic A × ACA F_1 mice but also in the parental A strain. Immunological tests showed that in these SEWA/fibroblast and TA3/fibroblast hybrids both groups of parental histocompatibility antigens were fully expressed. These findings thus eliminate the trivial objection that the suppression of malignancy seen in our experiments might simply have been due to mis-matching between the hybrid cells and the animals in which they were tested; but they do not eliminate a more sophisticated argument, which runs as follows. Since the progressive growth of the hybrid tumours *in vivo* obviously involves cell selection, might it not be possible that those cells that do grow progressively *in vivo* are cells that have lost the genetic determinants for the histocompatibility antigens of one parent or the other, or at least cells that express these antigens less strongly. An analysis of the segregation patterns of surface antigens in tumours derived from Ehrlich/A9 hybrid cells eliminates this possibility also.

The cells of the Ehrlich tumour, having been grown for several decades in mice of a wide variety of foreign genotypes, possess mechanisms that suppress or mask the histocompatibility antigens normally found on the cell surface (Hauschka & Amos 1957). When a cell in which the histocompatibility antigen complex is fully expressed is fused with the Ehrlich cell, the suppressive mechanisms of the latter continue to operate and the new histocompatibility antigens introduced into the hybrid cell are also suppressed. When A9 cells bearing the $H\text{-}2^k$ antigen complex are fused with the Ehrlich cell, this antigen complex can be detected in the hybrid cells only in very low concentrations or not at all (Harris *et al.* 1969). One might therefore suppose that if antigenic suppression were related to malignancy, these hybrid cells would grow readily in the test animal; but they are among the least tumorigenic hybrids that we have tested. The tumours arising from the injection of these hybrids are produced, as I have already described, by progressive overgrowth of segregants from which variable numbers of chromosomes have been eliminated. Analysis of these segregant tumours for the presence of the $H\text{-}2^k$ antigens donated by the A9 parent cell reveals that in some of them these antigens remain suppressed; but in others the $H\text{-}2^k$ antigens are fully restored (Klein, Gars & Harris 1970). These findings demonstrate that the absence of the $H\text{-}2^k$ antigens in the original Ehrlich/A9 hybrids was due to antigenic suppression and not to loss of the genetic determinants for these antigens; but they also demonstrate that the ability of the hybrid cells to grow progressively in the animal is not linked to the expression or suppression of the histocompatibility antigens. The factors governing the expression of these antigens and those determining progressive growth *in vivo* segregate independently. The Ehrlich cell can suppress other antigens introduced into the hybrid cell. The surface of the A9 cell bears antigens determined by resident viruses. These too can only be detected in low concentrations, or not at all, in Ehrlich/A9 hybrids; but, like the H-2 antigens, these virus-induced antigens may also reappear in some of the segregant tumours (Grundner *et al.* 1971). It is thus clear that malignancy, as defined by the ability of the hybrid cells to grow progressively in X-irradiated newborn syngeneic mice, is determined by mechanisms independent of those that determine the expression of either the histocompatibility antigens or the virus-induced surface antigens. The suppression of malignancy observed in our experiments and the differences in tumorigenicity between one hybrid cell and another are not explicable in immunological terms.

The general conclusions to be drawn from our investigations, despite the great variety of the experimental material, are clear. When a malignant cell is fused with a non-malignant one, or one of greatly reduced malignancy, the resulting hybrid cell, provided that it retains something close to the complete chromosome sets of both parent cells, has little capacity for growth *in vivo*. In our material the tumours that derive from the injection of such hybrid cells are produced not by progressive growth of the whole cell population injected, but by selective overgrowth of cells from which some chromosomes have been eliminated; and the evidence indicates that the generation of malignant variants in the hybrid cell population requires not merely an overall reduction in chromosome number, but the elimination of some specific chromosomes donated by the non-malignant parent cell. It is therefore difficult to escape the conclusion that in the hybrid cell the non-malignant partner contributes some factor or factors, linked to specific chromosomes, that can suppress the malignancy of the parental tumour cell or hold it in check. What then is the nature of this contribution?

It could be argued that normal diploid cells, or other non-malignant cells, contain specific suppressors of malignancy; but a less fanciful explanation becomes apparent if the possibility is considered that malignancy might ultimately be due to some lesion or lesions involving genetic (or stable epigenetic) loss. If the malignant phenotype were due to some form of genetic loss, or the synthesis of some non-functional gene product, then one would expect that this defect would be made good by a normal cell in which the relevant gene or genes are unimpaired. Complementation between the malignant and the normal cell would then be expected to restore the normal phenotype. Moreover, if more than one type of genetic loss, or impaired gene function, could determine a malignant phenotype, it should be possible in some cases to show complementation between different kinds of malignant cell: hybrid cells of non-malignant phenotype might then be produced from two malignant parent cells. We have therefore studied the properties of hybrids produced by fusing cells from two different sarcomas with lymphoma cells. The sarcomas (MSWBS and MBA) were induced by methylcholanthrene; the lymphoma (YACIR) by Moloney virus. The MSWBS and YACIR tumours have already been described. MBA is a solid transplantable sarcoma carried in CBA mice and bearing the H-2^k histocompatibility antigen complex. In order to obtain cell populations suitable for fusion, the solid tumour was explanted and the cells grown for several days *in vitro*. The MBA/YACIR and MSWBS/YACIR hybrids showed chromosomal constitutions corresponding closely to the sum of the two parental chromosome sets. (For the MSWBS/YACIR hybrids an almost exact correspondence could be established because the YACIR cells were essentially euploid and the MSWBS cells had a very tight mode and a number of marker chromosomes.) Individual clones of both hybrids were tested in the usual way in X-irradiated newborn syngeneic animals. The MBA/YACIR hybrids gave virtually 100% take incidences, comparable to those given by the two parent cells. In this case, complementation clearly had not occurred. Indeed, these hybrids provide the most decisive evidence that malignancy is not suppressed in some non-specific way by cell fusion itself. However, the MSWBS/YACIR hybrids gave substantially lower take incidences than the two parent cells, and the tumorigenicity of some of the clones was comparable to that of hybrids between the two parental tumour cells and A9 cells. This finding indicates that the malignant phenotype can in some cases be suppressed by fusing one malignant tumour cell with another. While this experiment does not prove that the genetic lesions responsible for malignancy involve losses of function, the results so closely mimic classical genetic complementation, that the idea, it seems to me, must be taken seriously. The difference in tumorigenicity between the MBA/YACIR hybrids and the MSWBS/YACIR hybrids might be due to differences in the stability of the two chromosome sets, or to different patterns of chromosome elimination under the selective pressures of growth *in vitro* or *in vivo*. But such a clear cut difference also suggests the possibility that the basic lesion responsible for malignancy might not be identical in the two sarcomas, even though both were induced by methylcholanthrene.

Whatever the precise nature of the genetic lesions responsible for malignancy, the present findings clearly delineate the operation of two processes in the formation of a malignant tumour: the generation of genetic variation in the cell population as a whole and selection by the body of certain specific variants that are capable of progressive growth. All our results can be summarized very simply in such Darwinian terms. The tumour cells used in our experiments are the end product of extreme selection; they have been selected for the ability to grow progressively *in vivo*. When such cells are fused with cells that have not been selected for this property (non-malignant cells), the malignant character of the tumour cells is not expressed in the hybrids, or is expressed much less strongly. In order to obtain a subpopulation of malignant cells from these essentially non-malignant hybrids, one must select again; and the frequency with which malignant subpopulations can be selected is a function of the degree of genetic variation in the hybrid cell population on which the selection operates. This view of the genesis of a malignant tumour does not require that an oncogenic agent should be highly specific in the genetic lesions that it induces. It would be enough if such an agent raised the level of genetic variation in the exposed cell population to a point where the specific lesions responsible for malignancy, although random events, had a high probability of occurring. Oncogenic agents such as chemical carcinogens or radiation would then act not by converting normal cells to malignant cells, but by generating enough genetic variation to permit malignant variants to occur with predictable frequency. This selectionist interpretation of malignancy implies, however, that the vast majority of the variants produced, and hence the vast majority of the chromosomal aberrations seen in pre-cancerous conditions, are essentially irrelevant to the production of the malignant state.

But what of oncogenic viruses? They obviously represent a genetic increment to the cell, not a genetic loss. And is it not reasonable to suppose that their role in the production of malignancy is a direct and specific one? The results that we have obtained with virus-induced tumours lead me to think differently. The SEWA tumour was induced by polyoma virus. The cells carry the virus and show the characteristic polyoma transplantation antigen. The YAC tumour was induced by Moloney virus. The cells also carry the virus and show the Moloney-specific surface antigen. When hybrids are made between these tumours and A9 cells, the hybrid cells continue to carry the oncogenic viruses of the parental tumour cells; they continue to show the specific surface antigens associated with the presence of these viruses; and they have, at least in some degree, the morphological features and growth patterns characteristic of virus-transformed cells. Yet SEWA/A9 and YAC/A9 hybrid cells, so long as they retain something like the complete parental chromosome sets, have little or no ability to grow progressively *in vivo:* the take incidences obtained with these hybrids are of the same order as those obtained with A9 cells. This, in my view, makes it unlikely that malignancy, as defined by progressive growth *in vivo*, is determined directly by some diffusible product of the viral genes. If it were so determined, one would expect malignancy to be dominant in these hybrid cells and not recessive, as is found to be the case. There is no doubt, of course, that some oncogenic viruses do produce direct effects on cellular morphology and behaviour: it has been shown, for example, that viral gene products induce changes in the surface properties of the cell (Benjamin & Burger 1970) and in the mechanisms that control the replication of the cellular DNA (Dulbecco & Eckhart 1970). But my interpretation of the significance of these effects in relation to the genesis of malignancy is that they generate the genetic instability, the genetic variation, on which selection for malignancy can operate. Some oncogenic viruses may well be very efficient in generating the right kind of genetic instability; but the genetic lesion or lesions that actually determine progressive growth *in vivo* might none the less involve functional losses even in tumours induced by viruses.

In conclusion, I should like to say something about the generality of our findings. If the fundamental lesions responsible for malignancy are indeed some form of genetic or epigenetic loss, then one would expect that in hybrids between malignant and non-malignant cells recessive inheritance of malignancy would be the rule. But our evidence on the nature of the genetic lesions is at best suggestive; and it may well be that a detailed examination of a wider range of experimental material will reveal cases in which malignancy is inherited as a dominant character. I do not believe that any such cases have yet been established; but their existence would not detract from the interest of the observations that I have described. That malignancy can be suppressed, and suppressed by the activity of a normal body cell is, it seems to me, no small thing; and perhaps I may be forgiven for hoping that the further exploration of this phenomenon may contribute in some small way to our understanding of what remains one of the most distressing of human maladies.

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ADDENDUM

The ideas put forward in the Croonian Lecture have since been explored by cytogenetic methods (*1*). The chromosome losses that take place in hybrids between malignant and normal diploid mouse cells are not random; particular chromosomes derived from the diploid parent cell are selectively eliminated. In certain crosses in which a detailed cytogenetic analysis was possible, it could be shown that the selective pressure was directed specifically against the two chromosomes #4 derived from the diploid parent cell. All tumors produced by these crosses had eliminated both copies of the diploid chromosome 4; however, both copies of the chromosome #4 derived from the malignant parent were retained. Specific selection against the diploid homologs of chromosome #4 could be demonstrated in crosses between diploid cells and two kinds of tumor, a melanoma and a fibrosarcoma. Analysis of crosses in which the two diploid chromosomes #4 were involved in cytologically recognizable rearrangements permitted more precise mapping of the genetic locus against which the selection pressure was directed: This locus was situated in the lower part of the upper half of chromosome #4. These studies permitted the conclusion that in the melanoma and in the sarcoma the activity of some critical locus on chromosome #4 was altered in some way.

The elimination of both copies of the diploid chromosome #4 from hybrids in which cancer had been suppressed was not, however, sufficient to induce the reappearance of the malignant phenotype in all cases; some hybrids from which both diploid chromosomes #4 had already been eliminated in vitro remained unable to generate progressive tumors. The reappearance of cancer in these hybrids evidently required the intervention of some additional event.

Although one or two apparent exceptions occur in the literature, the ability of diploid cells to suppress the malignant phenotype has now been convincingly demonstrated in a fairly extensive series of mouse cell crosses, and a similar effect has been found in crosses between malignant and normal diploid human cells (*2*).

Although crosses between malignant and diploid cells show little ability to grow progressively in vivo so long as they retain something like the complete chromosome constitutions of the two parent cells, crosses between different kinds of malignant cells usually remain malignant (*3*). If the cancer is determined by genetic defects that behave in a recessive manner, the common failure of different kinds of malignant cells to complement each other suggests the possibility that the same genetic locus often may be involved. Some support for this idea has recently been obtained from studies on the membrane proteins of hybrids in which cancer is suppressed and of segregant tumors derived from them. Malignancy, as measured by the ability of the cells to grow progressively in vivo, was intimately linked to the presence of a structural abnormality in the polysaccharide moiety of one particular membrane glycoprotein (*4*). This abnormality was present in a wide range of different tumors. It co-segregated with neoplasms in crosses between malignant and nonmalignant cells, and it remained linked to cancers in a stringent new test in which nonmalignant variants were selected from tumor cell populations by the use of a lectin.

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GUEST EDITORIAL

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Neoplasia: A Somatic Mutation or a Heritable Change in Cytoplasmic Membranes? [2]

THERE ARE few fields in biology where the literature on the theoretical and hypothetical aspects of a problem exceed that written about cancer. Since the disease affects virtually all multicellular organisms while exhibiting numerous and diverse characteristics, the theoretical proposals expounded as mechanistic explanations of neoplasia have often been based on the author's experiences in a relatively narrow area of scientific knowledge. The statement that each of us studying the basic mechanisms of neoplasia is convinced that his own concepts and theories of the genesis of this disease are the correct ones is eminently reasonable, since few workers waste their time in areas seeming to lack credibility. Yet even the most unusual theory, if it suggests experiments and questions, is worthy of scientific consideration and scrutiny. As our knowledge of biology advances, we find that theories long ago discarded may pose scientific questions that are reasonable in light of present knowledge.

Cancer as a disease is a horrible enigma: horrible because of the pain and suffering it causes; an enigma because, though we know a great deal about its biology, its genesis, and even its control, we know little of the basic molecular mechanisms of neoplastic transformation. Chemical carcinogens or their metabolites react with DNA and are usually mutagenic (*1–4*). They also react with RNA, protein, and other cell constituents (*2*, *4*, *5*). DNA viruses transform cells after integration of the virus nucleic acid into the cell genome (*6*, *7*), but revertant cells with integrated virus DNA exhibiting few if any properties of transformants in vitro are not infrequent (*8*, *9*). Most neoplastic cells, on continued growth, progress to aneuploid karyotypes (*10*, *11*); however, a few differentiate or revert to their normal, nonmalignant phenotype (*12*, *13*). Thus, though the cancer phenotype is apparently heritable and thought by many to reflect a specific or at least altered genotype, the exceptions to any proposed generalization are quite significant; in fact, the crucial targets for oncogens (chemical carcinogens, viruses, X-rays) are not known.

One of the more controversial facets of neoplastic disease (at least to some researchers) is the question of whether cancer is basically the result of an altered genotype or of a non-chromosome-heritable change in phenotype. It will probably be many years before this question can be satisfactorily answered for all neoplasms, but our present knowledge allows us to look critically at the riddle and to propose answers amenable to experimental verification.

GENETIC ORIGINS OF NEOPLASTIC TRANSFORMATION

Several lines of evidence support the concept that neoplasia is basically the result of a chemical alteration in the structure of one or more genes within the karyotype. Perhaps most outstanding is the obvious fact that when a cell exhibits biologic neoplastic properties, its progeny exhibit the same property to some degree. Such properties may be continued growth and replication, altered hormonal response, or excessive production of a cell product. However, some neoplasms may progress to a phenotype and genotype significantly different from those of the original neoplastic cell or tissue (*14*).

The genetics of neoplastic disease has been extensively studied in both rodents and man. Strains of mice specifically susceptible to certain types of neoplasms such as hepatomas (*15*, *16*), mammary carcinomas (*17*), or lymphoma (*18*, *19*) are well known. Recent studies have suggested that the latter disease in the AKR strain results from vertical transmission within the genome of the information contained in an oncogenic agent, probably viral (*20*). In man, several examples of neoplasms arising from a specific gene abnormality are known. Some retinoblastomas (*21*), familial polyposis of the colon (*22*), and bilateral medullary carcinoma of the thyroid (*23*), are all examples of neoplasms resulting from single, autosomal, dominant, genetic mutations. Several other genetic conditions in man predispose to specific types of neoplasia. Among these are xeroderma pigmentosum, Bloom's syndrome, and Chediak-Higashi disease. In contrast to the dominant mutations predominantly associated with a single neoplasm, these conditions are autosomally recessive. One of them, xeroderma pigmentosum, results from a defect in the repair of DNA. Similar mechanisms after DNA damage by chemicals or radiation have been studied (*24*, *25*). The proposal that neoplasia may result from damage to DNA and imperfect repair is a logical consequence of such experimentation (*26*), though at least one xeroderma patient showing normal repair still had numerous skin tumors (*27*).

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Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

Further evidence supporting the genetic nature of neoplastic transformation is the finding that virtually all chemical carcinogens, most of which in themselves are unreactive with macromolecules including DNA, RNA, or protein, must be converted to "ultimate" carcinogens with high chemical reactivity before they exert an oncogenic effect (*2*, *5*). Furthermore, most such agents in their active form are also mutagenic in several microbial and eukaryotic systems (*2–4*). Recent studies by Ames and his associates (*28*) used these metabolic conversions in a test system of liver microsomes to activate the potential carcinogen, and combined it with a strain of bacteria to show the mutagenic capability of the metabolic product(s).

Genetic information can now be experimentally introduced into cells to induce their neoplastic transformation. The nucleic acid from DNA oncogenic viruses is integrated into the host-cell DNA at an average of 5–60 molecules per cell genome (*29*). As a result of the discoveries of Temin and Mizutani (*30*) and Baltimore (*31*), integration of a DNA "provirus" into the host genome was demonstrated by Varmus and his associates (*32*) and Markham and Baluda (*33*). The oncogene hypothesis of Huebner and Todaro (*34*) proposes that one can find variants of cells transformed by either DNA or RNA oncogenic viruses, which are known to contain viral information but do not exhibit neoplastic properties. Under experimental conditions, suitable manipulation of several such revertants can bring out the neoplastic potential presumed inherent in the viral information inserted in the genome (*35*).

Certainly the most ubiquitous evidence that genome abnormality may be associated with the neoplastic transformation is seen in the numerous examples of abnormal karyotypes of neoplastic cells (*36*). Such abnormalities may be considered polygenic changes within the cell that are inherited by transmission from mother to daughter cell. At our present stage of knowledge, it appears that such karyotypic changes alter the dosage of those genes on the affected chromosomes. This may result in either an increase or decrease in the number of copies of a specific gene in the karyotype, though Ohno (*37*) has questioned the existence of viable neoplastic cells with less than the diploid content of DNA. As yet, except for possibly the Philadelphia chromosome seen in many patients with chronic myelogenous leukemia (*38*), no specific karyotypic pattern has been associated with neoplasia. More commonly, karyotype varies considerably in neoplasms arising from the same type of tissue within different individuals and sometimes even within the same individual (*37*, *39*, *40*). Recently Yamamoto et al. (*41*) demonstrated specific alterations in karyotypes of rodent cells transformed by polyoma virus. However, a significant number of neoplasms, especially in their early stages, do not exhibit any karyotypic anomalies when studied either by classical staining techniques (*42*, *43*) or by the newer banding methods (*44*). Therefore, though relatively irreversible genetic changes, as evidenced by karyotypic anomalies, likely exist in most mammalian neoplasms, the pathway by which the genetic anomaly originated is probably quite different for each case, depending on the host as well as the carcinogenic stimulus (*45*).

NEOPLASIA AS A MANIFESTATION OF AN EXTRAGENOMIC ALTERATION

The concept that neoplasia is not necessarily a result of structural alteration(s) within the genome is not new or unique. Before the era of molecular biology and our understanding of genetic mechanisms, the descriptive biology of neoplasia, promoted primarily by pathologists, suggested alterations in differentiation as one primary etiologic factor in cancer (*46*). A recent review considered our knowledge in this area in light of recent advances (*47*). Theoretical concepts, from both this laboratory and others, using models derived from studies in basic molecular biology, purported to explain neoplasia as an alteration in metabolic regulatory circuits (*48*, *49*).

Despite all these theoretical mechanisms, one must still accept the fact that whatever the result of the neoplastic transformation, it is clearly heritable. Therefore, any proposed mechanism of neoplasia must account for this phenomenon.

Differentiation as a Translational Function

To the pathology anatomist, the morphologic variation in human neoplasms, even in those derived from the same cell type, has been evident since the beginnings of cell pathology in the middle of the last century. With the advent of the electron microscope, the heterogeneity evident in the light-microscopic studies of neoplasms has been magnified. It is clear now that no single morphologic characteristic peculiar to all neoplastic cells has been, or probably ever will be, found.

Virtually all biochemical studies on mammalian neoplasms have in the final analysis supported the findings of the pathology anatomist on the extreme heterogeneity of both human and experimental neoplasia. Early investigations on the biochemistry of cancer demonstrated by glycolysis (*50*) and a number of other enzymatic capacities that cancer cells tend toward a single biochemical phenotype (*51*). However, data generated over the last two decades have shown that, when all experimental neoplasms of a specific class are studied, phenotypic heterogeneity is the rule rather than the exception. This includes data on hepatomas, mammary carcinomas, and myelomas (*52*). Furthermore, such phenotypic variability can be traced to those conditions considered preneoplastic. The heterogeneity is seen not only in the activity and regulation of enzymes within neoplasms but also in the stability of messenger RNA templates (*52*) and in the turnover characteristics of membranes of the endoplasmic reticulum (*53*).

In multicellular organisms, examples of varied, heritable, phenotypic expression are well known as the process of differentiation. In such a phenomenon, the fertilized egg, a single cell with a specific genetic content, gives rise to multiple phenotypically different clones of cells which breed true both morphologically and biochemically in vivo. When explanted to tissue culture, "dedifferentiation" of normal tissues often may occur.

Considerable evidence indicates that differentiation is the result of differential and sequential gene activation (*54–56*) possibly mediated through interaction of specific segments of the DNA with nonhistone proteins (*57*) or modified histones (*58*).

On the other hand, another characteristic of differentiation having its counterpart in the neoplastic transformation is that of changes in messenger RNA template stability during the differentiation process. Numerous examples of the acquisition of template stability for messenger RNA coding for proteins involved in specific differentiated functions have been described (*59*). Changes in messenger RNA template stability have been demonstrated in adult tissue undergoing specific phenotypic changes such as regeneration or normal maturation (*60*). Dustin (*47*) and Markert (*61*) have suggested that malignant transformation results from an abnormality in cell differentiation. Whereas messenger RNA template stabilization may be only a single parameter in the major processes of developmental biology and neoplasia, such cytoplasmic events are apparently associated with changes in the phenotype of the developing or transformed cell. Kafatos (*62*) has argued that lifetimes and turnover of messenger RNA template may well be the major mechanisms of cell differentiation. If both differentiation and neoplasia result from altered template stability, then those highly malignant tumors demonstrating rapid growth should possess extremely stable templates for those messenger RNA's whose products are directly involved in cell replication.

Reversion of Neoplastic Transformation

While one popular dogma of oncology is that the neoplastic transformation is irreversible, a significant number of examples of reversion of the neoplastic process to a normal or quasi-normal state have been reported. One of the best known examples of such reversions or "redifferentiation" is seen in the plant system fully described by Braun (*63*). However, recent evidence (*64*) has questioned the interpretation of those experiments. Furthermore, plant cells may undergo an extragenomic heritable cell change known as habituation, which can occur spontaneously and results in altered phenotypes in the cells undergoing the change (*65*). In the newt, epidermoid carcinomas produced by chemicals are reversible, once the carcinogenic stimulus is removed (*66*). In the mammal, the mouse teratoma (*13*) is the best experimental example of redifferentiation of neoplastic cells. Here, as in the plant, single cloned neoplastic cells may exhibit a reversion of their biologically malignant potential to produce essentially normal tissues obeying the growth restraints and homeostatic mechanisms of the host in which they live. In the human, a classic example of such reversion to a fully differentiated state is seen in a small percentage of neuroblastomas, a highly malignant neoplasm of childhood which is almost always fatal (*67*). Sometimes, however, either in response to some therapeutic measure or unknown stimulus, a neuroblastoma undergoes differentiation to morphologically normal adult neurons, a process also occurring when the neuroblastoma cells are explanted in vitro.

Thus in a small but significant number of instances, a neoplastic transformation reversible in vivo demonstrates that all the biologic characteristics of neoplasia can occur in a cell and then be lost from the same cell at a rate exceeding by far the rate of any spontaneous mutations.

Studies from the laboratory of Sachs (*68*), Heidelberger (*69*), and DiPaolo et al. (*70*) demonstrated that neoplastic transformation in cultured cells can be induced by chemicals, viruses, or high-energy radiation. Sachs showed that a highly significant number of neoplastic transformations can be reversed and the cells can lose some or even all the phenotypic characteristics of the transformed state. Extending these studies, he and his co-workers also demonstrated that most of these revertants show polyploid or near polyploid karyotypes (*71*). From this, they proposed that through such additional genetic information, reversion suppresses the transformation characteristics (*68*).

The recent descriptions of nuclear transplantation from renal tumors of the frog into ova with subsequent development of tadpoles, exhibiting no evidence of neoplasia (*72*), also argue that the phenotypic changes in this neoplasm are extragenomic, despite the addition by a herpesvirus of new genetic information into the original neoplasm (*73*).

Template Stability and Membrane Configuration

Accepting the initial premise of this section that neoplasia is a result of heritable extragenomic changes in the cell, we are still confronted with the question of their mechanism. Theoretical considerations from this laboratory proposed that messenger RNA stabilization results from the interaction of the messenger RNA template with intracellular membrane systems (*47, 74*). This membrane-RNA interaction is thought to involve an association between those proteins closely associated with messenger RNA and proteins of the surface of the endoplasmic reticulum, the outer nuclear membrane, or the internal surface of the plasma membrane. The theoretical requirements for such an association were extensively treated in a recent review (*75*). The mosaic characteristics of cell membranes are well documented (*76*). The relationship of the polysome to the macromolecular mosaic pattern of the membrane forms a structure that has been termed the membron (*75*).

The principal question involves the heritability of this membrane mosaic. From the model of the inheritance of the external membrane pattern of certain protozoa (*77*), specific membrane mosaic patterns may act as their own templates in further membrane synthesis (*74*, *75*). Altered mosaic patterns induced either by chemicals, virus synthesis, or radiation damage would then be inherited in subsequent generations. Since new membrane synthesis occurs during specific times of the cell cycle (*78*), one would expect that the altered pattern would be "fixed" into the new membrane of daughter cells during a single cell cycle. The phenomenon of fixation in the induction of neoplastic transformation and its probable need for a complete cell cycle are now well documented from both in vivo and in vitro studies (*79*).

Theoretically, a heritable membrane change could give rise to a heritable phenotypic change based on messenger RNA template stabilization. Such a model could explain several phenomena such as differentiation, antibody diversity, and the production of embryonic antigens by neoplastic cells (*75*). The principal difficulty in such a concept in relation to cancer is that it does not account for the obvious genetic changes occurring in established, growing neoplasms. The final section of this paper will attempt to resolve the 2 viewpoints into a single picture of the mechanism of neoplastic transformation.

MEMBRANES AND CHROMOSOMES IN THE NATURAL HISTORY OF NEOPLASIA

Our knowledge of the biologic development of cancer in vivo stems from the original investigations and subsequent proposals of Berenblum (*80*), Boutwell (*81*), and Salaman and Roe (*82*) on the initiation and promotion of neoplasms in their normal biologic environment. These concepts have not changed, though in more recent studies on in vitro neoplastic transformation it is not so easy to distinguish the two or more stages recognizable in the whole animal.

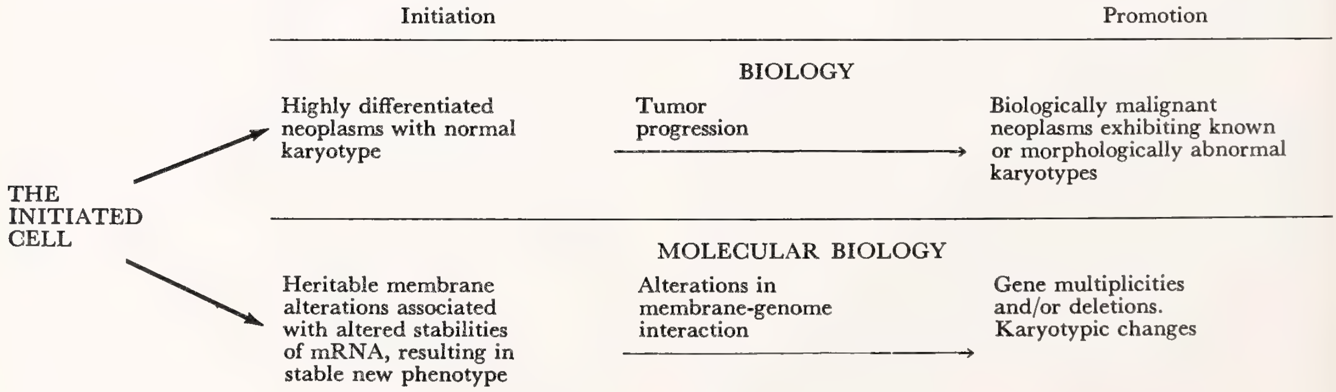
The direct evidence for genomic alteration(s) or somatic mutation(s) associated with, if not causally related to, neoplastic transformation is supported principally by the abnormal karyotypes characteristic of most established neoplasms (*37*) and the addition of viral information during transformation by such agents. That most chemical carcinogens are mutagens supports more indirectly a genetic basis for the malignant transformation. On the other hand, many neoplasms expressing a high degree of differentiation, exhibiting relatively slow growth rates, and generally being in the class biochemically characterized by Potter as minimal deviation tumors (*83*) appear, by even the most modern techniques, to have a normal chromosomal constitution (*44*). The evidence for reversion of the neoplastic to the normal or quasi-normal state has also been obtained with well-differentiated neoplasms both in vivo and in vitro (*66*, *67*). The studies on the alteration of messenger RNA template stability and the membron concept were formulated primarily from experiments done with minimal deviation hepatomas.

Thus, the natural history and pathogenesis of neoplasia in vivo may well relate to genetic constitution. Although the genetic and the epigenetic theories of neoplasia initially appeared to be at odds, the two can now be reconciled in relation to our knowledge of the natural history of cancer.

Initiation and Promotion: Membrane Alteration and Genomic Multiplicity, Respectively

Berenblum's original concepts (*80*) argued that the phenomenon of tumor initiation was probably irreversible, whereas tumor promotion could be modulated. Roe et al. (*84*) recently showed, however, that, in the mouse skin system, initiation by hydrocarbons is not totally irreversible (*84*). The fact that certain neoplasms early in their natural history may undergo a reversion or differentiation both in vivo and in vitro affirms that the initiation of neoplasia is an immutable process. However, modulation of the neoplastic phenotype and genotype during the phase of promotion is the rule of essentially all established neoplasms.

The scheme shown in text-figure 1 relates the classical phases of neoplastic progression, those of initiation and promotion, to the genetic and epigenetic concepts discussed thus far. In this hypothesis, a class exists of neoplasms that are the direct progeny of the theoretically initiated cell (*85*). To this class


Initiation
Promotion
BIOLOGY
Highly differentiated neoplasms with normal karyotype
Tumor progression
Biologically malignant neoplasms exhibiting known or morphologically abnormal karyotypes
THE INITIATED CELL
MOLECULAR BIOLOGY
Heritable membrane alterations associated with altered stabilities of mRNA, resulting in stable new phenotype
Alterations in membrane-genome interaction
Gene multiplicities and/or deletions. Karyotypic changes

TEXT-FIGURE 1.—Pathogenesis or natural history of neoplasia.

of transformed cells belong those neoplasms exhibiting no gross karyotypic abnormalities but having heritable phenotypes altered from those of their normal precursor cells, with such change resulting from modification in the stability of messenger RNA templates. Alteration of the templates would result from changes in the membrane mosaic structure of the cell, inherited by the mechanisms discussed earlier (*76*, *77*). In these cells, the membrane alterations producing heritable phenotypic changes may not lead to the enhancement of growth potential seen in grossly visible neoplasms. However, these changes may permanently alter the phenotype of the cell such as has been described in relation to the feeding of hepatocarcinogens to rats (*86*). The existence of non-neoplastic aneuploid cells has been known for some time (*87*, *88*).

In the natural history of neoplasia, most highly differentiated neoplasms exhibiting enhanced growth potential would be expected to progress to the promoted state. The promoted cell, as defined in text-figure 1, is biologically malignant and has karyotypic changes resulting from gene multiplicities or deletions, predominantly the former (*37*). In accord with this is the fact that metastases, the sine qua non of the malignant as opposed to the benign neoplasm, are almost always aneuploid (*39*, *89*). The importance of gene dosage effects in biologically malignant neoplasms was discussed earlier in this paper in reference to the work of Yamamoto et al. (*41*) and Sachs (*68*). Recently, Gandin (*90*) also proposed theoretical concepts related to neoplastic transformation and gene dosage. Many neoplasms produced by carcinogenic agents apparently can exhibit karyotypic abnormalities in their earliest definable cell populations. This fact demonstrates that the initiated state as defined in text-figure 1 may be bypassed, depending on such factors as the dosage of the inciting agent and its general effects on the cell.

What is the relationship between changes in the macromolecular mosaic of intracellular membranes in the initiated state and the aneuploidy or gene multiplicities and/or deletions characteristic of the promoted cell? The association between membrane and cell replication, though fairly clearly delineated in prokaryotes, is still unclear in the eukaryotic cell (*91*, *92*). However, evidence supports a relationship between the nuclear membrane and interphase chromosome structures (*93*, *94*). Therefore, it seems reasonable to argue that the transition from the initiated to promoted state may be related to heritable changes in membrane structure which can in turn alter the normal sequences of chromosome DNA synthesis and mitosis within the replicating cell.

CONCLUSIONS

With our present knowledge, no attempt to generalize a mechanistic explanation for a biologic phenomenon is satisfactory. The theses presented here attempt to relate apparently divergent concepts of neoplastic transformation to a single picture correlating both the natural biologic history of the pathogenesis of neoplastic development in vivo with the presumed critical biochemical or molecular biologic changes associated with this pathogenesis. Until we fully understand in molecular terms the biology of neoplasia as expressed by the disease in the living animal, we will not have accomplished the principal goal of experimental oncology, and even then we may still be unable to control completely the manifestations of this disease in the human.

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ADDENDUM

Although no experimental evidence has come to light that would negate the concepts presented in this article, an extension of our knowledge of the natural history of neoplasia in certain systems, especially teratomas, has required some refinement and extension of the proposal presented in text-figure 1. Such an extension was published in 1977 following a symposium on the stability of the neoplastic state (*1*).

The studies by Mintz and Illmensee (*2*) and Papaioannou et al. (*3*) demonstrated that cloned teratoma cells, which were diploid or near diploid, could be transplanted into developing blastocysts, and the resultant stable chimeric mouse resulting from such a combination exhibited no neoplasms. On the other hand, if aneuploid teratoma cells were inoculated into blastocysts, teratomas and other neoplasms developed in the neonatal and adult chimera (*1*). These studies suggested that an initiated cell population, as indicated in text-figure 1, may possess a normal genotype but express the neoplastic phenotype and, under certain circumstances, is fully capable of reverting to the normal phenotype even after expressing biologic malignancy. Such reversion is apparently dependent on a cellular environment comparable to that found in the developing fetus. On the other hand, initiation may result immediately or within a few cell divisions in a cell and/or clone with an abnormal genotype which is then incapable of reversion to "normalcy" under almost all conditions.

Therefore, although reversion of biologically malignant neoplasms to benign or normal cell populations remains an extremely rare event, it is conceivable that initiated cell populations dormant or unrecognized in otherwise normal individuals may be induced to revert to normalcy so long as damage to the genome has not occurred. Application of this concept to populations-at-risk for specific neoplasms resulting from environmental factors remains a subject for future consideration.

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GUEST EDITORIAL

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Relationship of the Selective Metastatic Behavior of Tumors of Reticular Tissues to the Migration Patterns of Their Normal Cells of Origin

MUCH of the work published in both the scientific and popular literature suggests that malignant tumors are radically different from the tissues from which they arise. While the effect on the victim may be quite drastic, this may be due to a small alteration in the cell that ultimately develops into a malignant tumor. That this might be especially true of tumors of the reticular tissues was suggested by Wallace (*1*), who states: "Many epithelial tumors may acquire marked invasive capacities only very gradually, perhaps by selection. These properties may not reach a high level during the life of the host, and experimental tumors may become highly invasive only after many transplant generations. By contrast, tumors of hematopoietic tissues, lymphoid tissues, and trophoblast have inherited these properties from their normal cells of origin, and as a consequence, fall, as a rule, into the incurable group of neoplasms. Tumors of this type perhaps possess a clinical malignancy out of line with their stage of development or autonomy as otherwise evaluated." Since tumor cells inherit properties from their normal cells of origin, possibly tumors of the reticular tissues also inherit their metastatic preference.

Tumors of the reticular tissues have selective metastatic preferences. Neoplasms of phagocytic cells (macrophages or histiocytes) metastasize regularly to the liver (*2–4*); plasma cell tumors metastasize to the bone marrow (*5, 6*); Hodgkin's-like tumors metastasize either to the spleen or lymph nodes (*4*); and lymphocytic tumors metastasize to all lymphatic organs (*4*). This selective metastasis was believed to represent differential growth of tumors in different organs (the congenial-soil hypothesis of Paget). A Hodgkin's-like reticulum cell neoplasm, which metastasizes selectively to the spleen, afforded an opportunity to test the congenial-soil hypothesis. Equal numbers of tumor cells were injected into the spleen (the preferred site) and the kidney (a nonpreferred site). Contrary to expectations, the tumor did not have a higher mitotic index in the spleen, though the tumor mass there was greater than in the kidney. This neoplasm metastasized to the spleen after implantation into the kidney. From these experiments I concluded that the tumor metastasized selectively because tumor cells in the spleen tended to remain there, whereas those in other organs tended to migrate out (*7*). This was confirmed by Parks (*8, 9*) who followed radioactively labeled tumor cells. The finding that selective metastasis is more likely a result of differential cell migration rather than differential growth suggests a basic change in how these metastatic patterns should be viewed: If these tumor cells migrate selectively, then one can postulate that this ability to selectively migrate may be a vestige of the

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Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

original migrant behavior of the normal cell of origin. Should this hypothesis be tenable, much information about the migration patterns of the normal reticuloendothelial system might be gleaned from studying the metastatic behavior of transplantable tumors of the reticular tissues.

The purposes of this communication are to: 1) briefly review the available evidence for the hypothesis that selective metastasis may be a vestige of the migration pattern of the normal cell of origin; and 2) indicate the potential value of transplantable reticular tumors as tools for the study of the function of cells of the normal immune system.

Lymphocytes and Lymphocytic Tumors

The literature on lymphocytic tumors is massive and has been reviewed by Dunn (*4*). Two basic metastatic patterns are generally found: One type involves the circulation of many leukemic tumor cells and the eventual infiltration of all organs, whereas the second type produces metastasis in all lymphoid organs without leukemia. This behavior is so characteristic that it is rarely discussed in the literature.

Gowans and Knight (*10*) clearly demonstrated that small lymphocytes circulate in the bloodstream, go from the bloodstream into lymphatic tissues, and reenter the blood vascular system via the lymphatics.

The tendency of tumors to produce leukemia appears to be directly proportional to the circulating propensities of the normal cell of origin. This concept is supported by the behavior of myeloid leukemia (*11*), which almost invariably produces a leukemia state.

Plasma Cells and Plasmacytomas

Plasma cell tumors in mice originate in the soft tissues. They occur spontaneously in C3H mice and are associated with chronic ulceration of the intestine at the ileocecal junction (*12–14*). They have been induced in BALB/c mice by the intraperitoneal introduction of mineral oil (*15*, *16*) or lucite (*17*, *18*). When these tumors are transplanted, they metastasize selectively to the bone marrow (*5*, *6*). The etiology of plasmacytoma in man is unknown, but metastasis to the bone is its most consistent feature.

The evidence that non-neoplastic plasma cells migrate to the bone marrow is only suggestive. They are normally found in small numbers in the marrow and their numbers can be increased by antigen administration (*19*).

Gengozian *et al.* (*20*) demonstrated that ". . . bone marrow cells transplanted into irradiated animals showed definite antibody-forming potential when marrow was obtained from presensitized donors, but lacked the capacity if normal nonsensitized donors were used." It is attractive to postulate that the bone marrow acquires this antibody-forming potential from the migration of plasma cells.

Reticulum Cells and Reticulum Cell Sarcomas

Marshall (*21*) pointed out that the principal difficulty faced by a morphologist is the fact that the nuclei of various cells of the reticular and connective tissues look the same. It is impossible to tell, by observing the bean-shaped nucleus with an open chromatin pattern, whether this "undifferentiated" cell is destined to become a macrophage, fibroblast, or will divide to form more "undifferentiated" cells. The cytoplasm provides information about cell function only after the cell has differentiated. There is probably a variety of physiologically distinct cells, each having specific fates which are not morphologically discernible. When tumors of the reticular tissues are maintained in transplant, further classification, based on their metastatic pattern, is possible. We have, to date, been able to distinguish three distinct types of "reticulum cell" tumors.

One is a histiocytoma that appears to be erythrophagocytic and accumulates hematoidin pigment (*3*, *22*). Smears of this tumor, stained with Romanowsky-type dye, show that the predominant cell resembles the monocyte (having a convoluted nucleus and a dusty cytoplasm). This tumor has been classified by Dunn (*4*) as a reticulum cell sarcoma type A. On transplantation, these tumors metastasize to the regional node of the transplantation site and to the liver. Metastasis to the liver always occurs (*2*, *22*). Metastasis to the lung is rare, though the tumor cells must pass through the lung to reach the liver. We have implanted this tumor directly into the lung and find that it has limited growth, with the animal succumbing to massive metastasis of the liver.

A second type of tumor resembles Hodgkin's disease. It contains a mixed population of large reticulum cells and lymphocytes. Dunn (*3*, *23*) calls this tumor a type "B" reticulum cell neoplasm. Some debate has ensued as to whether this lesion is the murine equivalent of human Hodgkin's disease. Since the histologic picture in Hodgkin's disease is highly variable, one would not expect the few transplantable mouse tumors to have the exact histologic picture of the typical classic case of Hodgkin's disease. The morphology and the metastatic pattern are similar enough to allow us to equate the experimental findings in the mouse with human Hodgkin's disease. Two distinct metastatic patterns are evident in the transplantable tumors: One transplantable tumor always metastasizes to the spleen and the regional lymph nodes, whereas the other metastasizes to all lymphoid organs except the spleen. The metastatic patterns are retained through serial transplant. In our experience the lymphocytes disappear on serial transplantation and only the reticulum cells remain.

A third type of tumor consists of reticulum cells that tend to form multinucleate giant cells. Our single transplantable line of this tumor metastasizes selectively to both the liver and kidney. This tumor appears to have changed its metastatic pattern during transplantation, with one line now metastasizing to the spleen.

The metastatic pattern of transplantable histiocytoma correlates with the migration pattern of normal histiocytes. Roser (*24*, *25*) and Russell and Roser (*26*) used histiocytes from various sources. After being labeled (by their ingestion of radioactive colloidal gold *in vivo*), the cells were injected intravenously into syngeneic mice. Their migration was followed by determination of the total amount of radioactivity in each organ and by radioautography. The results clearly show that histiocytes ingesting colloidal matter will migrate to the liver. Over half the radioactivity injected ended up in the liver, regardless of the source of the histiocytes. The metastatic pattern of histiocytoma correlates well with the migration pattern of the normal cell of origin.

To compare the Hodgkin's-like tumor with its normal cell of origin, one must first determine what the normal cell of origin is. The tumor consists of a mixture of cells found in chronic inflammation. This similarity to chronic inflammation is so striking that Hodgkin believed this neoplasm to be a form of tuberculosis. The isolation of various lines of transplantable reticulum cell tumors affords an opportunity to study the cell types and their interrelationships. The availability of large populations of cells allows us to answer a number of questions: What is the relationship of the lymphocyte to the tumor? Are the lymphocytes produced by the tumor (or vice versa), or do these cells migrate into the tumor from the host? How many different types of reticulum cells are there, and what is the relationship of one type to the other? Detailed experimental studies of these tumors might clear up the confusion presently existing in our understanding of the nature of these cells.

Labeled antigen is picked up at sites of inflammation and can be found in "large macrophages and multinucleated cells" in the lymph nodes as long as 9 months after injection (*27*). The tendency of Hodgkin's-like tumors to metastasize to the lymph nodes or spleen suggests that the tumor cells might be the neoplastic counterpart of the antigen-retaining cells. It has been established that 2 cell types are necessary to form antibody (*28*). One of these cells apparently is the small lymphocyte, and the other a cell that will adhere to glass and which has been referred to as a "macrophage." Perhaps this Hodgkin's-like tumor may help to elucidate the nature of this "macrophage."

Conclusions

The retention of normal function by neoplastic tissue is basic to the identification of tumors. Skin tumors continue to cornify; intestinal tumors continue to produce mucus and enzymes; tumors of endocrine tissues continue to produce hormones, etc. There is little reason to suspect that reticular cells that have given rise to a tumor would have most of their basic properties altered during the neoplastic transformation. It is more reasonable to expect that the carcinogen has changed some mechanism involved in the birth-death equilibrium; any alteration which increases the birth rate of cells relative to the death rate, or decreases the death rate relative to the birth rate, will result in tumor. In reticular cells whose function can be morphologically or chemically observed, there has been no indication of any loss of function when they have become neoplastic. Tumors of histiocytic cells continue to phagocytize (*3*, *22*) and tumors of plasma cells continue to secrete immunoglobulins (*29*). There are tumors of the reticular tissues that consist of cells whose function is unknown. These tumors may be considered to be the neoplastic counterpart of cells having a normal function in the animal. Study of the behavior of these tumors can provide clues to the nature of their normal cells of origin.

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ADDENDUM

The Science Citation Index has, to date, four references to this Editorial. All support the thesis that the metastatic patterns of reticuloendothelial tumors correspond to the migration patterns of the normal cells of origin of the tumors.

Barker and Waksal (*1*), who studied the metastatic patterns of thymus-derived lymphocytes in leukemia of AKR mice, found that leukemia cells taken from the thymuses of leukemic mice had metastatic patterns different from those taken from the spleen, which, in turn, varied from those taken from the lymph nodes. They concluded that "The resultant disease patterns correlated with the homing patterns of these T-cell populations. These differences were further reflected in the differential reactivity of these leukemic T-cells to mitogens and the T-dependent antigen, sheep erythrocytes."

Fuks et al. (*2*) studied the behavior of human non-Hodgkin's lymphoma following treatment with radiation. They concluded that "The patterns of migration for subclasses of lymphocytes are unknown, although it is possible that such phenomena may exist. Similar modes of migration have been postulated, based on some experimental evidence, for malignant cells of lymphoreticular origin (Pilgrim, 1972). Such a hypothesis implies that a malignant lymphoma cell originating in a lymph node and travelling through the blood stream may preferentially enter and lodge in another lymph node, even one remote from its site of origin, rather than in the bone marrow, liver or other extralymphatic site. Thus, it is consistent with our data on the relative frequency of extensions confined to lymph node bearing areas, including those in remote, noncontiguous sites to which the tumour cells presumably spread *via* the blood stream."

Cossman et al. (*3*) found a large population of complement receptor B-lymphocytes in nodular sclerosing Hodgkin's disease. It is still not known whether these lymphocytes are neoplastic cells or an invasion of normal lymphocytes into a malignant reticulum cell stroma. The disappearance of the lymphocytes from transplanted murine Hodgkin's-like neoplasms indicates that the lymphocytes may be neoplastic cells. Until this question has been resolved, any conclusions about the relationship of the cells in Hodgkin's disease to their normal cell of origin remains highly speculative.

In a second paper, Cossman et al. (*4*) demonstrated that the cells of nodular and diffuse, poorly differentiated lymphocytic lymphoma are derived from B-lymphocytes of the follicular center type. The nodular lymphomas preferentially involve the follicular areas of the lymph nodes, lending support to the thesis that the metastatic pattern of a tumor corresponds to the site of migration of the normal cell of origin.

A transplantable reticuloendothelial tumor consists of a large population of a type of cell found in small numbers in the normal animal. The neoplastic transformation presumably involves a change in the rate at which cells are born relative to the rate at which they die. In other respects, the behavior of these cells are probably unchanged. Therefore, these tumors are valuable tools for the elucidation of the mysteries of the immune system. With the exception of plasmacytoma, which has been used for the study of antibody synthesis, these tools remain largely unexploited.

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GUEST EDITORIAL——**by Frank Lilly**

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Mouse Leukemia: A Model of a Multiple-Gene Disease [1,2]

IN MAN, existing evidence that certain lymphoid cancers are influenced by the genetic constitution of the individual is incomplete and less than totally compelling. In fact, where we *are* with respect to genetic studies of the human leukemias and lymphomas is reminiscent of where we *were* with respect to mouse leukemia [3] a (human) generation ago. Genetic studies of this murine disease, emanating from the laboratories of Furth and MacDowell and others, had indicated rather strongly that host genetic factors are of considerable importance in leukemogenesis, but the number of genes involved and their mechanisms were entirely unknown.

It is possible that certain types of human leukemia or lymphoma will prove to be largely due to single recessive genes with quite incomplete penetrance. More likely, however, is the hypothesis that these diseases occur as a function of the individual's constitution at several gene loci, each of which makes a contribution to susceptibility or resistance to the disease. As with most biologic phenomena, the phenotype (the individual's risk of the disease) is determined by the interaction of the host genotype at these multiple loci and certain environmental factors, such as irradiation and benzene inhalation, which can increase the innate risk of the disease.

Our understanding of the inheritance of many single-gene diseases is relatively advanced, but the same cannot be said for multiple-gene diseases. Quantitative genetics has long offered us ways of describing multiple-gene traits in terms of their population dynamics. But, for an understanding of the mechanisms whereby a complex genotype influences a biologic character, there seems to be no substitute for the lengthy process of identifying, mapping, and studying one by one the separate genes which act concertedly in each individual.

The Friend Virus Disease

The disease induced in mice by Friend Virus (FV) (*1*) is a model of a neoplastic condition, susceptibility to which is a multiple-gene trait. Our understanding of the genes controlling the host's response to FV has recently increased enormously, and thus the disease might serve as a model for other multiple-gene conditions. The FV disease is perhaps not ideal as a model for spontaneous leukemia. It has been called a "laboratory disease," for it occurs, if at all, only as a minority type among spontaneous leukemias of mice. Still,

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[1] This work was supported by Public Health Service contract 65-612 within the Special Virus-Cancer Program of the National Cancer Institute.

[2] This article is based, in part, on the extensive review "Genetic Control of Murine Viral Leukemogenesis" by Frank Lilly and Theodore Pincus, which will appear in Advances in Cancer Research, vol. 17.

[3] The term *mouse leukemia* is used in its general and traditional sense, thereby including several morphologically and histologically dissimilar disease entities.

Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succintly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

an understanding of how several genes act, each in a different way, to influence the response of mice to this virus might be useful in suggesting ways of looking at the spontaneous disease. Four categories of genes influencing the host response to FV are summarized in table 1.

The characteristic FV disease is an erythroleukemia the outstanding feature of which is a logarithmic increase in spleen weight during 7–10 days beginning almost immediately after virus inoculation. This splenomegaly is due to the proliferation of erythrocyte precursor cells. At about 2 weeks, the growth of the spleen levels off and may even decrease somewhat prior to death, which occurs in most mice during the 4th or 5th week of infection. At lower virus doses, this splenomegaly may reverse itself, so that some hosts regain a normal spleen size, either transiently or permanently. This tissue regression suggests the participation of the host immune system in resistance to the disease, and, in fact, the rapidly proliferating cells in the spleens of the diseased mice show a new, virus-specified surface antigen, Friend-Moloney-Rauscher (FMR), to which mice can be induced to make antibodies.

FV is a C-type RNA tumor virus. Susceptible mice display viremia detectable within 3–4 days and rapidly attaining a level of up to 10^6 infectious units per ml. The major assays of the virus have been based on host spleen weight (*15*) or, more recently, on the ability of many strains of FV to induce macroscopic foci on the surface of the spleen 9 days after intravenous virus inoculation (*16*). Among other advantages of this spleen focus assay is that it reflects to only a minor degree the host immunologic response to the virus inoculation.

Repeated passage of FV in rats (or in mice of certain resistant genotypes) leads to the loss of the erythroid cell specificity of the virus, which now induces a disease similar to the lymphoid neoplasms induced by wild-type (Gross) leukemia virus (*17*). The virus recovered from rats no longer induces spleen foci, which suggests that this property of FV was due to a separable component lost from the complex population of viruses constituting FV (*18*, *19*). This component of FV complex was called spleen focus-forming virus (SFFV); the rest was called lymphatic leukemia virus (LLV). A major property of SFFV is its tissue specificity for erythroid precursor cells.

SFFV is defective, i.e., it appears incapable of carrying out its entire infectious cycle and of inducing erythroleukemia in the absence of coinfection with LLV. Thus LLV serves a helper function, supplying factors missing from the SFFV genome, which permit the production of infectious SFFV virions (*20*).

The host response to SFFV and to LLV are governed separately by two genes, *Fv-2* and *Fv-1*, respectively (*2*). The biologic manifestations of these two host regulatory genes are so different in character that it is immediately clear they must operate by separate mechanisms. Let us consider them separately.

Fv-2.—Susceptibility to SFFV is regulated by *Fv-2*, a gene first identified and named *Fv* by Odaka (*3*). Mice homozygous or heterozygous for the *Fv-2*^{s} allele are highly susceptible to SFFV; mice homozygous for the recessive *Fv-2*^{r} allele are, by contrast, highly resistant to the SFFV in most strains of FV complex (table 2). However, a variant strain of SFFV has been described (*21*) which can induce spleen foci even in *Fv-2*^{r} hosts.

Fv-2 has been mapped in linkage group II of the mouse genome. Although no gene with developmental effects in erythroid cells has been mapped

TABLE 1.—*Host genes affecting the response to FV in mice*

Gene	Effect on—	Systems studied
Fv-2	Spleen focus-forming virus (SFFV) component of FV.	FV (*2*, *3*).
W, *Sl*, *f*	Hematopoietic cells (hereditary anemias).	FV (*4–6*).
Fv-1	Helper virus component of FV complex.	FV (*2*), naturally occurring murine leukemia viruses (MuLV's) (*7*, *8*).
Rgv-1 (*H-2*)	Immune response to virus-associated antigens.	Gross virus (*9*, *10*), BALB/Tennant-leukemia virus (B/T–L) (*11*), FV (*12*), mammary tumor virus (*13*), radiation leukemia virus (RadLV) (*14*).

TABLE 2.—*Effect of Fv-2 on response to FV*

Fv-2 genotype	Phenotype
$Fv\text{-}2^s/Fv\text{-}2^s$ -----	Highly susceptible to focus formation by SFFV.
$Fv\text{-}2^s/Fv\text{-}2^r$ -----	
$Fv\text{-}2^r/Fv\text{-}2^r$ -----	Highly resistant to focus formation by SFFV.

in this region, it is intriguing to speculate that *Fv-2* might show such effects, even in the absence of SFFV infection. If *Fv-2* is expressed uniquely in cells of the erythroid series, then this restricted tissue distribution of the $Fv\text{-}2^s$ gene product might be the basis for the erythroid tissue specificity of SFFV. Three genes known to give rise to hereditary anemias in mice, *W* (*4*), *Sl* (*5*), and *f* (*6*), have been studied in this respect and found to decrease susceptibility to SFFV, apparently by decreasing the availability of target cells for the virus.

The critical event in the infectious cycle of SFFV, which fails to occur in $Fv\text{-}2^r$ hosts, is unknown. That susceptibility to the virus is completely dominant in this system might suggest an analogy with the control of the host range of RNA leukosis-sarcoma viruses in chickens by the *tva* and *tvb* genes (*22*), which govern the penetration of the cell membrane by the virus. However, in this chicken virus system there is a well-defined relation between the serotype of the virus and its host range; this relationship has not been described for any murine leukemia virus system, so that the analogy between *Fv-2* and the chicken genes may well prove misleading. The cell-surface alloantigen system, *H-7*, appears to map in the same region of linkage group II as *Fv-2* (*23*), but it is not yet known whether these two genes are identical or merely linked to each other.

Probably *Fv-2* is no factor or only a minor one in governing the response of mice to leukemia viruses other than SFFV, such as Gross virus and LLV. C58 mice show a high, spontaneous incidence of leukemia and B10·BR mice are highly susceptible to Gross virus leukemogenesis, but both these strains are homozygous for $Fv\text{-}2^r$. Strains of MuLV show the same growth patterns in the XC cell assay in embryo cells of congenic DBA/2 and D2.R^B mice, which differ at the *Fv-2* locus (*7*).

Fv-1.—Susceptibility to the helper component of FV complex (LLV) and to many other strains of MuLV with similar properties is strongly influenced by the host's genotype at the *Fv-1* locus. *Fv-1* has not yet been located in the mouse genome, but it is not appreciably linked to *Fv-2* or *H-2*. Two alleles at the *Fv-1* locus are known, $Fv\text{-}1^n$ and $Fv\text{-}1^b$, and mouse strains are said to be N-type /or B-type, respectively, depending on their *Fv-1* genotype. The helper component of the original strain of FV complex is N-tropic (*24*), meaning that it is highly infectious in N-type mice (e.g., DBA/2) but poorly infectious in B-type mice (e.g., BALB/c). A strain of FV complex produced artificially has a B-tropic helper component, so that this complex is much more infectious in B-type than in N-type mice (Lilly, unpublished). N-tropic FV complex can be adapted by repeated forced passage in B-type mice to a stable genetic form called NB-tropic (*25*), meaning that it is equally infectious in N- and B-type mice (i.e., it is no longer sensitive to the *Fv-1* type of its host).

Susceptibility to both N- and B-tropic strains of helper is recessive in $Fv\text{-}1^n/Fv\text{-}1^b$ heterozygotes. However, these hybrids show the same level of susceptibility to NB-tropic virus strains as the parental N- and B-type mouse strains, an indication that NB-tropic viruses are not merely mixtures of N- and B-tropic viruses.

The resistance to helper virus conferred by *Fv-1* type is relative, whereas resistance to SFFV due to *Fv-2* type is very nearly absolute. The mechanism of this relative resistance is reflected in the titration patterns of the viruses in mice of different *Fv-1* genotypes. N-tropic viruses show one-hit, dose-response patterns in N-type mice, (susceptible), whereas they show multiple-hit patterns in B-type mice and in F_1 progeny of N-type × B-type crosses (relatively resistant); B-tropic viruses show one-hit patterns in B-type mice (susceptible) and multiple-hit patterns in N-type and hybrid mice (relatively resistant). Thus, at extremely high virus doses, N- and B-type mice differ relatively little in their responses; at low doses, the two types of mice may show 100- to 1000-fold differences in their responses. Table 3 summarizes the relation between virus tropism and host *Fv-1* type.

A similar effect of host *Fv-1* type was seen in studies of MuLV strains in mouse embryo cells in vitro with the XC cell assay (*8*). Naturally oc-

TABLE 3.—*Effect of Fv-1 on titration patterns of FV by the spleen focus assay in mice**

Fv-1 genotype	Phenotypes		
	"Hitness" of dose-response curve with FVs of different tropisms		
	N-tropic (F-S)	NB-tropic (F-B)	B-tropic
$Fv\text{-}1^n/Fv\text{-}1^n$	One-hit	One-hit	Multiple-hit.
$Fv\text{-}1^n/Fv\text{-}1^b$	Multiple-hit	One-hit	Multiple-hit.
$Fv\text{-}1^b/Fv\text{-}1^b$	Multiple-hit	One-hit	One-hit.

*Mice homozygous for $Fv\text{-}2^r$, being resistant to spleen focus-formation, cannot be used for *Fv-1* testing.

curring MuLV isolates are either N- or B-tropic in this system, again depending on the *Fv-1* type of the mouse embryo cells used. As in the spleen focus assay in vivo, relative resistance of the host cells reflects multiple-hit responses, by contrast with the single-hit responses in highly susceptible cells (*26*). In contrast with the spleen focus assay, however, the XC assay produces results to which neither the *Fv-2* type of the host cells nor the presence or absence of SFFV in the virus preparation appears to be relevant. Thus the spleen focus assay detects a combined effect of SFFV and its helper virus, whereas the XC assay detects an effect of the LLV component of FV complex.

The multiple-hit pattern of the titration of N-tropic FV complex in B-type mice has been used as the basis for the detection of helper activity for SFFV in preparations of leukemogenic viruses other than LLV (*27*). These SFFV-free viruses, when mixed with low doses of the N-tropic FV complex, augment the production of spleen foci in B-type hosts by providing supplementary helper virus activity, which tends to "correct" the multiple-hit titration pattern to a single-hit pattern. Helper activity in this system is a property of many strains of N- and NB-tropic leukemogenic viruses, and such activity has also been associated with certain avian and feline RNA tumor viruses and also in extracts of leukemic tissues of avian, feline, and even human origin [(*28*) and Steeves, unpublished data]. In some of these cases, it is not clear that the helper activity of the preparations is necessarily due to the presence of a virus; nevertheless, the detection of the activity exclusively in tissues of leukemic origin and/or in tissues infected with RNA tumor viruses is striking.

The molecular basis for the single- or multiple-hit pattern of helper viruses in different hosts is purely a matter for speculation. One implication of the multiple-hit pattern in a virus titration is that the focal event detected is due to simultaneous infection of a cell with multiple helper virus particles. Whether these multiple particles are members of a single population of identical particles or consist of one each from multiple populations of different particles is not known. Multiple-hit patterns are, as noted, dominant in *Fv-1* heterozygotes. Biologic phenomena governed by dominant genes are usually due to an activity related to the protein encoded by this allele; the corresponding recessive phenomena are usually due to the absence of an active form of the gene product. From this generality it may be inferred that the gene products of the $Fv\text{-}1^n$ and $Fv\text{-}1^b$ alleles either are themselves, or govern the synthesis of, repressors of B- and N-tropic viruses, respectively, since their activity converts potentially single-hit patterns (high susceptibility) to multiple-hit ones (relative resistance). Whether the potential repressor substance is already present in the cells prior to virus infection or is made in response to the stimulus of virus infection is unknown.

Although formal experimental proof is still lacking, there are strong indications that *Fv-1* genotype is a major factor in susceptibility to some other viral tumors in mice, including spontaneous leukemia. F_1 crosses of high-leukemic AKR mice ($Fv\text{-}1^n$) with a low-leukemic mouse strain tend to show a relatively high or low incidence of spontaneous leukemia, depending, to a first approximation, on whether the low-leukemic parent contributed the $Fv\text{-}1^n$ or $Fv\text{-}1^b$ allele, respectively. Furthermore, although the tissues of such F_1 progeny always show some MuLV activity in the XC assay, the level of this virus activity tends to be about as high as that in AKR mice if the F_1

animals are homozygous for *Fv-1*^{n}, whereas the level tends to be at least tenfold lower if the progeny are *Fv-1*^{n}/*Fv-1*^{b} heterozygotes (*29*).

Thus the two separate genes, *Fv-1* and *Fv-2*, must both be taken into account if one wishes to predict that a given strain of mice will be susceptible to infection by FV. Both these genes influence by different mechanisms what might be referred to simplistically as the "virologic phase" of the disease syndrome, regulating the ability of the complex virus to infect host cells, to replicate in them, or to transform them.

Rgv-1.—Another host gene influences the host response to FV in an entirely different manner. This gene has been partially mapped within the *H-2* genetic region of linkage group IX of the mouse. Having been first identified in studies of susceptibility to Gross virus leukemogenesis (*9*), it received the symbol, *Rgv-1* (resistance to Gross virus) (*10*).

Rgv-1 influences the FV disease by affecting quantitatively the splenomegalic response to the virus (*12*). This effect appears relatively late in the development of the disease, since it is only minimally detectable in the 9-day spleen focus assay for the virus. However, mice of the congenic BALB·B strain (BALB/c-*H-2*^{b}) require 10 or more times as much FV for the induction of splenomegaly at 14–21 days by comparison with BALB/c (*H-2*^{d}) mice (*30*). Furthermore, BALB·B mice are much more likely to recover from FV-induced splenomegaly than are BALB/c mice receiving the same virus dose.

These characteristics of the action of *Rgv-1* are consistent with the hypothesis that the fundamental effect of the gene is on the "immunologic phase" of the FV disease, i.e., the immune response to antigens associated with virus infection. Two different but not mutually exclusive mechanisms have been postulated to explain this immunologic effect.

1) There exists in the same subregion of *H-2* to which *Rgv-1* has been localized another gene, *Ir-1*, which regulates the level of the immunologic response to a variety of natural and synthetic antigens (*31*). This suggests that the strength of the immune response to virus-specific antigens might also be regulated by *Ir-1*, and that resistance to FV as determined by *Rgv-1* thus represents a stronger response to a given antigenic stimulation by FV, as determined fundamentally by *Ir-1.*

2) The expression of the FV-induced FMR antigen on the surfaces of infected spleen cells appears to be influenced by a gene in the *H-2* region (*30*). In the BALB/c-BALB·B pair of congenic mouse strains, the antigen appears simultaneously in both hosts and attains the same maximum level of expression around day 7–11 after virus inoculation. Thereafter, however, the cells of the relatively susceptible BALB/c mice show progressively lower levels of FMR until, during the 4th week, they are often almost devoid of detectable antigen expression, whereas the antigen remains at high levels throughout the disease in the more resistant BALB·B hosts. The mechanism of this genetically determined antigen disappearance is unknown. Possibly, anti-FMR antibodies are in some way responsible for the loss of the antigen, either by simply masking it or by a more complex mechanism analogous to the modulation of thymus-leukemia (TL) antigens on thymic leukemia cells (*32*). Whatever this mechanism may be, it is reasonable to suppose that the disappearance of the antigen in BALB/c mice is causally related to their greater susceptibility to the disease. To the extent that the antigen fails to persist in these mice, their immune response is ineffective in controlling the disease by elimination of the virus-infected cells.

Rgv-1 appears to influence the host response to virtually all RNA tumor viruses of mice, presumably by a similar mechanism in each case. Experiments have shown an association between *H-2* type and susceptibility, not only to FV and Gross virus but also to B/T-L virus (*11*), RadLV (*14*), and even the mammary tumor virus (*13*). In all these cases, the *H-2*^{b} allele conferred relative resistance to the neoplastic disease, whereas the *H-2*^{k} and *H-2*^{d} alleles were associated with susceptibility.

Spontaneous Leukemia

Two of the three genes discussed thus far as representing the major genetic components of the FV disease syndrome are also significant in the genesis of spontaneous leukemia. *Fv-1* has already been discussed in this respect. *Rgv-1* influences the spontaneous disease by a correlation of its incidence with *H-2* type in AKR × (AKR × C57BL) backcross mice (*10*) and also by a comparison of its relative incidences in congenic AKR and AKR-*H-2*^{b} mice (*33*).

Other genetic factors associated with spontaneous leukemia in mice are currently under study in several laboratories, and these show promise of enabling us to go much further in elucidating the natural history of mouse leukemia. The leukemogenic virus (or some component of it) endogenous to high-leukemic AKR mice can be detected by the XC cell assay in vitro, and Rowe (*34*) has found that the inheritance of this virus in crosses with virus-negative strains is due to two independent dominant genes, V_1 and V_2, neither of which is closely linked to *Fv-1* or to *Rgv-1* (*29*). Taylor et al. (*35*), examining crosses of AKR and low-leukemic C57L mice for the occurrence of leukemia virus-associated group-specific-1 (gs-1) antigen, demonstrated the existence of what seems likely to be the same two genes, V_1 and V_2. Whereas it is not certain that these genes represent chromosomal sites of the virus genome (as opposed to genes regulating the expression of a viral genome located elsewhere), it nevertheless seems likely that these genes will be shown to be a factor in spontaneous leukemogenesis.

In addition to gs-1, 2 other antigens associated with spontaneous AKR leukemias are under study: Gross cell surface antigen (GCSA) and G_{IX} (*36*). The patterns of inheritance of these 2 antigens are quite complex, and the G_{IX} antigen requires 2 genes for its expression. The possible relation between these 2 genes and the V_1 and V_2 genes is currently under investigation. The eventual relationship of these antigens to the occurrence of spontaneous leukemia is, at present, problematic, though it cannot be doubted that further immunogenetic studies will contribute to an understanding of the natural history of leukemia viruses.

Mention should also be made of the intriguing but still unexplained influence of the *hr* (hairless) gene on leukemogenesis in HRS/J mice, a highly inbred strain which, for technical reasons, has been kept segregating at the *hr* locus. Phenotypically hairless mice (*hr*/*hr*) of this strain show a high incidence of spontaneous leukemia, whereas their normal-haired littermates (+/*hr*) have only a low incidence of the disease (*37*). Both hairless and normal-haired mice, like AKR mice, are uniformly homozygous for *Fv-1*^{n}, *Fv-2*^{s}, and *H-2*^{k} (*38*), and both types of mice appear to carry leukemia virus. Thus the effect of *hr* (or of a closely linked gene) seems to be exerted at a relatively late stage in the genesis of the disease.

It seems evident that the genetic approach to mouse leukemia offers a tool with enormous powers of resolution for the study of this disease. However, if leukemia presents such a complex genetic picture in mice, where the availability of many diverse inbred strains makes the elucidation of the problem feasible though tedious, what then are the prospects for such an approach to the human disease?

One approach is to look for genes homologous to those known in other species, and this approach is already being attempted in at least one case. Since so many parallels are already recognized between the human leukocyte antigen (HL-A) system and *H-2* in the mouse, it is natural to wonder if HL-A might be associated with a factor influencing human leukemia. The results of studies in several laboratories in recent years have shown a significant—though far from complete—correlation between the occurrences of certain HL-A antigens and Hodgkin's disease (*39–41*). An analogous correlation has sometimes been found for leukemias or lymphomas (*42*), though here the evidence to date is less convincing (*43*). If several genes are important in human lymphoid tumorigenesis, then correlations of the diseases with any one of the genes should be only partial in such epidemiologic studies.

Obviously, any candidate for a "human leukemia virus" that is transmissible in tissue culture and for which a simple assay is available could be the object of a study of its growth patterns in cells of different individuals, and such a study could reveal one or more genes important in human lymphoid tumorigenesis. Such studies will certainly be carried out soon after the advent of the necessary techniques. Genetic experiments capable of resolving the individual genetic factors in mouse leukemia could not be conducted until the discovery of techniques for working with leukemia viruses.

What might be the advantages of knowing the genes in man which lead to an increased risk of leukemia? After all, at this time there is little in the way of helpful advice that one could offer to persons suspected of being "leukemia-prone." We should recall that, if we know of genes which lead to increased susceptibility to leukemia in mice, it is because these genes have alleles favoring resistance to the disease. If the genes can favor resistance, we can try to find out how they do it, and then perhaps we can do it, too.

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GUEST EDITORIAL —————————————— **by Leonard Weiss** [1,2]

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Neuraminidase, Sialic Acids, and Cell Interactions

MANY CONFLICTING and often unrelated statements have been made about the physiologic role of sialic acid moieties bound to the cell periphery and their effect on cellular interactions. Also of current interest is the modification of cell interactions after removal of cell surface sialic acids by incubation with neuraminidase. In this review, I will trace the background of some of the work and attempt to clarify some apparent contradictions.

A survey of the literature unfortunately makes necessary the obvious statement that cell surface "glycopeptides" are not synonymous with "sialic acids." This review concerns some effects of neuraminidase and sialic acids on cell interactions and not the much larger topic of cell-surface mucopolysaccharides and the enzymes degrading them.

Cell Surface Sialic Acids

The detailed biochemistry of sialic acid moieties in the mammalian cell and its periphery is covered in a number of excellent reviews, including those by Cook (*1*), Ginsberg and Neufeld (*2*), Roseman (*3*), and Winzler (*4*).

The sialic acids are *N*, *O*-acetylated derivatives of D-neuraminic acid, which has not been found in living organisms. Neuraminic acid is a C9 amino sugar, which occurs in cells as a pyranose. Three sialic acids commonly found in nature are the *N*-acetyl-, *N*-glycolyl-, and 7-*O*-acetyl- (*5*). At present, only the *N*-acetyl and *N*-glycolyl (*6*) derivatives of neuraminic acid have been observed in the cell periphery.

Oligosaccahrides are structural determinants in a number of antigens with the genetic control mediated through specific glycosyltransferases. Although sialic acids are involved in the MN antigen system, their precise role has not yet been determined. A function for sialic acids in histocompatibility antigens is also suggested by the observation that *N*-glycolylneuraminic acid specifically inhibits some mouse alloantisera (*7*). As far as I am aware, sialic acids have not been specifically associated with the antigenic-determinant sites of tumor-specific antigens or haptens, of the glycoproteins or glycolipids associated with the cell surface.

Much of the current interest in sialic acid moieties at the cell periphery stems from the observation by Burnet et al. (*8*) that filtrates from *Vibrio comma* (*V. cholerae*) and *Clostridium perfringens* (*C. welchii*) destroyed the receptor sites for influenza viruses at the surfaces of human erythrocytes. In 1950, Ada and Stone (*9*) demonstrated that the receptor-destroying enzyme (RDE) from *V. comma* reduced

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Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

the net surface negativity of human erythrocytes by more than 80%, and suggested that the material enzymatically cleaved from the surfaces of these cells contained acidic groups. In 1958, Klenk (*10*) suggested that acylated neuraminic acids could contribute to the negative charge on erythrocytes; this suggestion was enforced by Ada and French's (*11*) purification of RDE and its identification as neuraminidase, which specifically hydrolyzes the glycosidic bond joining the keto-group of *N*-acetylneuraminic acid to a sugar or sugar derivative (*12*). It is now known that *V. comma* neuraminidase (3.2.1.18) is a mucopolysaccharide, *N*-acetylneuraminylhydrolase, which cleaves the 2,3 and 2,6 glycosidic linkages between terminal *N*-acetylneuraminic acids and mucopolysaccharides. A final step in the erythrocyte "saga" is due to Cook et al. (*13*), who demonstrated an 80% reduction in the net surface negativities of human erythrocytes incubated with highly purified neuraminidase, and showed by chemical analysis that this reduction was associated with the liberation of free sialic acid moieties from the cells.

The presence of sialic acids at the surfaces of tissue cells was first demonstrated by an in vitro detachment procedure. In these experiments, highly purified neuraminidase significantly facilitated the detachment of cells cultured adherent to glass plates compared with controls, after application of accurately reproducible shearing pressures (*14*). Sialic acids in the peripheries of many different types of cells have since been demonstrated, either by electrokinetic methods in which loss of cell surface net negativity follows neuraminidase treatment and/or by analysis of supernatants for free sialic acids after treatment of whole cells or their isolated surface membranes with the enzyme (*4*, *15*). Since sialic acid moieties are resistant to release from glycolipids by neuraminidase (*10*, *16*) such analyses will lead to underestimates of sialic acids at the cell periphery.

Dynamic Aspects of Sialic Acids in the Cell Periphery

In common with other constitutents of the cell periphery, the sialic acid content is variable during mitosis and growth. In some cultured cells, the contribution of neuraminidase-susceptible anionic sites to net surface negativity increases at the G2 phase of the mitotic cycle (*17*, *18*); in others it does not (*19*). This difference may also depend on culture techniques. The amounts of sialic acids liberated from CHO cells by neuraminidase, expressed in terms of calculated unit cell surface area, were fairly constant during the cell cycle. From this observation, Kraemer (*20*) suggested that variations of the type observed by Mayhew, and Brent and Forrester, were due to change in cell surface conformation rather than change in amounts of sialic acids present. As measurements of electrophoretic mobility provide an index of net surface charge density at the hypothetical slip-plane, or electrokinetic surface of cells, and as it is quite impossible to estimate true cell surface area by measurements of cell diameter, it is difficult to follow Kraemer's arguments.

Later work by Glick et al. (*21*) with KB cells demonstrated an increase in their total sialic acid content at about the time of mitosis. These observations cannot be directly related to those of Kraemer, since the sialic acid moieties he studied were released by neuraminidase and do not, therefore, include those associated with membrane glycolipids which are not susceptible to neuraminidase. However, such sialolipids are expected to be a minor source of membrane sialic acids. Quite apart from differences in experimental techniques and expected inherent differences between cell types, the whole question of metabolism and turnover in the cell periphery is extremely complex. Warren and Glick (*22*) interpret their own results on cultured mouse fibroblasts to indicate that the rates of synthesis of surface membranes are approximately similar in growing and nongrowing cells. However, in the growing cell, the material is incorporated into the membrane complex, and little turnover is exhibited. In contrast, in the nongrowing cell, newly synthesized material is incorporated, with a corresponding loss of existing membrane substance and a resulting high turnover without net change in content.

Sialic Acids in the Cell Periphery and Malignancy

Sialoproteins.—The relationship between sialic acids in the cell periphery and malignancy is not as clear cut as once commonly thought. The pioneering work of Ambrose and his colleagues (*23–25*) revealed that cells from stilbestrol-induced hamster kidney tumors and from hepatomas induced in the rat by dimethylaminoazobenzene had higher surface negativities than their normal analogues. When cultured hamster fibroblasts were converted by polyoma virus, they fell into two recognizable types in culture. One had electrophoretic mobilities indistinguishable from the original fibroblasts, whereas the other showed an increase in neuraminidase-susceptible mobility. This latter observation was consistent with the findings of Defendi and Gasic (*26*) that polyoma-induced conversion of embryonic hamster BHK cells was accompanied by the acquisition of a dense, pericellular, neuraminidase-susceptible region of Hale-positive, acid mucopolysaccharide. In addition, Fuhrmann (*27*) observed that, whereas the electrophoretic mobilities of both proliferating liver cells and those from an ascites hepatoma were higher than normal liver cells, only the surface negativity of the malignant cells was reduced by incubation with neuraminidase.

In common with others (*28*, *29*), Fuhrmann (*27*) therefore suggested that malignancy is associated with sialic acid-mediated increases in cell surface charge density. This reasonable working hypothesis, which would have simplified the interpretation of a number of cell interactions pertinent to malignancy, has not withstood test by experiment. Thus in a series of electrokinetic studies on a wide range of normal and neoplastic cells of human origin, Vassar et al. (*30*) observed that mesenchymal tumor cells had higher net surface negativities than carcinoma cells, that incubation with neuraminidase caused a greater loss of negativity from the surfaces of mesenchymal tumor cells than carcinoma cells, and that normal and malignant epithelial cells did not have significantly different surface charge densities. Other electrokinetic studies on human and animal tumors have also failed to support the hypothesis (*31–33*). The progression to a more virulent form of the rat ascites hepatoma AS–30D was accompanied by a decrease of 27% in neuraminidase-labile cell-surface sialic acids (*34*). In addition, decreased amounts of sialic acids have been reported in strains of fibroblasts (*35*, *36*) following in vitro transformation; and Grimes (*37*) has shown that simian virus 40 (SV–40)-transformed cells have only 60% as much sialic acid as "normal" 3T3 cells, and that, following transformation with SV40 virus, BALB/c cells lose 65% of their sialic acids.

Grimes also made the important observation that the reduced levels of sialic acids were associated with reduction in sialyltransferase activity. These enzymes catalyze the transfer of sialic acids during sialoglycoprotein synthesis (*38*). At present, therefore, there appears to be no consistent relationship between sialoglycoproteins and malignancy. There are apparent discrepancies between estimates based on the release of free sialic acids from whole cells treated with neuraminidase and estimates based on changes in electrophoretic mobilities after incubation of cells with the enzyme (*39*); such discrepancies have been discussed at length (*15*, *141*). The sialic acid content of cell peripheries can be determined definitively only by chemical analysis of isolated surface membrane complexes. The main value of the electrokinetic approach is that it probably gives a reasonable idea of the *average* charge density "sensed" at the surface of one cell by another and the contribution of neuraminidase-susceptible sialic acids to this. As the cell periphery is a three-dimensional structure, sialic acids can be located in it at different depths. The further away from the electrokinetic surface of a cell an ionized group is, the less will be its projected contribution at the plane of zeta-potential. However, it is currently impossible to precisely quantitate three-dimensional charge density and distribution from electrophoretic mobility data. Both techniques have a place in relating membrane structure to function and should be regarded as complementary rather than mutually exclusive methods of study.

Sialolipids.—Sialic acids are present in membrane glycolipids (*40*), and changes in these moieties with respect to malignancy are relevant here. An important initial observation made by Hakomori and Murakami (*41*) was that BHK 21 cells transformed by polyoma virus have less *N*-acetyl-neuraminyl-lactosylceramide than untransformed cells. They originally reported that in the transformed line this deficiency was balanced by a corresponding increase in glycolipids with "incomplete" oligosaccharide chains (e.g., lactosylceramide). However, later publications (*42*, *43*) report that, although the fall in hematoside levels was found consistently, corresponding increased levels of the "incomplete" lactosylceramide or glucosylceramide were not. More recent and definitive studies by Hakomori et al. (*44*) on whole cells show that chick embryo fibroblasts contain 4 types of sialoglycolipids, SL–1 (hematoside), SL–2 (disialohematoside), SL–3, and SL–4. After infection with Rous sarcoma viruses, the levels of SL–2 and SL–3 fell profoundly in parallel with the development of neoplastic transformation; SL–1 levels fell after transformation was completed; precursors of these sialolipids accumulated in the transformed cells, and in these cultures the turnover rates of SL–1, SL–2, and SL–3 were accelerated.

The levels of these sialoglycolipids, which almost disappear on or after malignant conversion, increase in cells exhibiting contact inhibition of growth, with increased amounts of cell contact (*45*). The enzymatic basis for the fall in sialoglycolipid levels has been described in a preliminary report (*46*) of the decrease in UDP-galactose: lactosylceramide α-galactosyltransferase activity following transformation of NIL–2E and BHK cells by polyoma virus. The investigations described above by Hakomori and his colleagues, and by others, were made on whole cells as distinct from purified membrane preparations. The studies of Weinstein et al. (*16*, *47*), together with their careful review of the literature, indicate significant variation in the type and amount of sialolipids found in the surface membranes of various types of cells. In surface membranes isolated from L cells, glycolipids accounted for only 0.7% of the total lipids, and only the hematosides and disialoganglioside were found in these preparations.

Although these isolated surface membranes contain approximately 6 times as much sialic acid per mg of protein as whole L cells, only 27% of whole-cell sialic acid was recovered in the ganglioside fraction, compared with 21% in the surface membrane preparations. In addition, Weinstein et al. (*16*) note that in L cells, ceramidelactoside and monosialoganglioside appear to be restricted to intracellular membranes. Thus, analyses of whole cells cannot be regarded a priori as a useful indication of the sialolipid content of their surface membranes. More work on isolated surface membrane preparations is required to clarify the possible relationship between sialolipids in the cell periphery and malignancy.

Topography of Neuraminidase-Susceptible Groups at the Cell Periphery

In an attempt to explain the lack of correlation between measurements of cellular electrophoretic mobility and cell contact and adhesion, it was pointed out that measurements of electrophoretic mobility give an index of *average* net surface charge density, but that cell contact occurs between small parts of adjacent cell surfaces and is not, therefore, an average phenomenon. Thus it was argued that filopodial or other low radius of curvature projections from one cell possibly make contact with regions of lower-than-average charge density on another (*48*). Recently, this hypothesis of the electrical heterogeneity of the cell surface has been tested on human erythrocytes (*49*) and Ehrlich ascites tumor cells (*50*, *51*). The densities and location of positively charged colloidal iron particles adsorbed to the surfaces of glutaraldehyde-fixed control cells and those previously incubated with neuraminidase have been studied by electron microscopy, and the results correlated with estimates of the cell surface charge densities. In both types of cells, the neuraminidase-susceptible sialic acids, which account for approximately 60% of the net negativity of erythrocytes and 24% of Ehrlich ascites cells, appear to be arranged in high-charge density clusters, in which an average of (very) approximately 30 anionic sites are packed into an area of not more than 80 nm^2.

It was postulated that anionic sites resistant to neuraminidase or ribonuclease were spread in a more homogeneous manner over the cell surface. Recent statistical analyses have revealed that the colloidal iron hydroxide particles are distributed at random along the length of sections. From a knowledge of section thickness, distribution statistics, and the coordinates of the particles, their two-dimensional distribution patterns can be plotted by the computer (*52*). When composites based on a number of sections are made, lower-than-average charge density areas large enough to accommodate 100 nm-diameter filopods from other cells are seen. Whether antigenic determinant sites at the cell surface bear any constant spatial relationship to these postulated high-charge density zones is difficult to establish. The validity of present techniques for seeing these antigenic sites by the use of ferritin-conjugated antibody (*53*) has been cast in doubt by the work of Davis (*54*). The relationship of the zones to concanavalin A receptor sites as demonstrated by Nicholson (*55*), and other sites, is also speculative.

Peripheral Sialic Acids and Associated Factors

If the amount, and/or arrangement of sialic acid moieties in the cell periphery are somehow directly related to cancer, then whether these hypothetical quantitative and/or qualitative differences are causally related to the interactions of malignant cells should be considered. Although, as will be discussed, some surface properties and cell interactions appear to be related to their sialic acid content, it is possible that, from the viewpoint of causal relationships, changes of this type in the cell surface may be of less functional importance than underlying associated changes. For example, the synthesis of sialoglycolipids and sialoglycoproteins reflects, among other factors, the status of "multiglycosyltransferase" systems.

Although it has been proposed that specific adhesions can occur between cells when the glycosyltransferases at the surfaces of one cell "recognize" their glycosidic substrates at the surface of another (*3*), direct evidence in support of this hypothesis is lacking. In the case of sialyltransferases and cell surface sialic acids, experimental evidence does not reveal loss of specific adhesive properties in cells incubated with neuraminidase. In addition, as proposed by Watkins and Morgan (*56*), blood-group genes code for specific glycosyltransferases, and treatment of erythrocytes with enzymes of this type can convert O into A, and B into AB blood groups (*57*). It does not seem too speculative to suggest that the glycosyltransferases released from one cell can change the surface antigenicity and interactions of other cells, normal and malignant.

Neuraminidase and Immunogenicity

Any agent altering the reactivity of cell surface antigens to humoral or cellular antibodies may do so by direct and/or indirect actions. Neuraminidase may therefore act directly by removing sialic acid moieties from antigenic sites themselves, thereby fundamentally changing their relevant chemistry. The enzyme may produce indirect effects on sites adjacent to the antigenic determinant groups on the same or nearby molecules and, as a consequence of removal of ionized sialic acid carboxyl groups, the antigens may become more or less susceptible to antibodies. Many examples of the effect of charge on the association of antigenic determinant sites with antibodies are given by Pressman and Grossberg (*58*). As stated earlier, sialic acids do not appear to be important tumor-specific antigenic determinants; therefore, if neuraminidase treatment alters the antigenic reactivity of a cell periphery, it is probably the result of indirect, rather than direct, actions.

Immune mechanisms in which lymphoid cells and macrophages bring about contact-mediated lysis of malignant cells are thought to be important in the clinical course of cancer. On the assumption that contact between the surfaces of killer and target cells would be promoted by treatment of the killer cells with neuraminidase, two types of experiments have been reported in the literature: 1) transplantation experiments and 2) tumor regression experiments. In critical comments on interpretation of this work it should be emphasized that the reported observations themselves are not in doubt, but that the potential significance of the work warrants such detailed appraisals.

Transplantation experiments.—In these experiments, tumor cells with or without prior incubation with neuraminidase were injected into animal hosts and the frequency of subsequent tumor development was noted. For example, Sanford (*59*), working with TA3 cells, and Currie and Bagshawe (*60, 61*), with Landschutz ascites tumors in mice, reported that fewer tumors developed in the animals receiving neuraminidase-treated tumor cells than in the controls. These findings were contrary to those of Ruhenstroth-Bauer et al. (*29*) with an ascites hepatoma and Hauschka et al. (*62*) with the identical TA3 and hosts used by Sanford (*59*), since neither of these groups observed any effect of neuraminidase treatment on subsequent tumor takes.

To account for the totally contradictory observations of Sanford and themselves, Hauschka and colleagues reexamined their experimental techniques. Both groups used neuraminidase dissolved in acetate buffer at pH 5–6. Whereas Hauschka et al. diluted neuraminidase to a final concentration of 25 U/ml in phosphate-buffered saline, final pH 7.1, Sanford utilized another diluent for the enzymes with a final pH of 5.2. For controls Hauschka et al. incubated tumor cells in phosphate-buffered saline at pH 7.1, whereas Sanford's cells were at pH 6.6. In comparable experiments both groups incubated cells in suspensions containing only 3×10^4 cells/ml. With such low concentrations, Hauschka et al. note that many samples of cells had to be examined by the standard trypan-blue viability test to assess cell viability accurately. These tests must be done on neuraminidase-treated samples with the same cell density as those injected into the animals, since it is well known that at low concentrations (e.g., 3×10^4 cells/ml) and in the absence of adequate amounts of protective proteins in the medium, cell survival rates are significantly less than at greater cell densities (e.g., 10^6–10^7/ml).

Examination of these variations in technique showed (*62*, text-fig. 2) that with the procedures used by Sanford only 30% of the cells were viable after 30 minutes' incubation with enzyme at a concentration of 3×10^4 cells/ml. If 3000 TA3–M cells from this batch were injected into mice 30 and 77% of the A/Ha and C3H/He animals, respectively, survived after neuraminidase treatment, compared with 13 and 91% in the respective controls. These findings are expected, since the LD100 of TA3 for A/Ha (syngeneic) mice is only 35–40 tumor cells compared with 3,000 cells for C3H/He allogeneic mice. When TA3–M cells were preincubated at densities of 10^7 cells/ml with a negligible loss of viability, injections of 3,000 neuraminidase-treated cells resulted in 0 and 37% of survivors in A/Ha and C3H/He mice, respectively, compared with 0 and 22% in the controls. In experiments of this type, knowledge of the LD100 or LD50, in relation to the numbers of cells injected, is mandatory. In the absence of such knowledge simplistic statements about whether tumors develop or not are meaningless in terms of the viability of the inoculum. For example, in A/Ha mice injection of 4,000 TA3 cells is expected to produce 100% takes if only 1% of the cells are viable.

In the light of these experiments and considerations, although Hauschka et al. were able to repeat Sanford's results, they could not agree with her interpretation that the reduced transplantability of neuraminidase-treated TA3 cells was due to "unmasking" of histocompatibility antigens. Moreover, as they found no difference in the H–2 antigenic titers of TA3 cells before and after neuraminidase treatment, they concluded that, at least in the case examined by them, a decrease of in vivo tumor takes, particularly if seen in syngeneic hosts, implies nothing more than a reduction below the LD100 level in the number of viable cells inoculated, rather than an increase in their surface antigenicity. This loss of viability was probably due to incubation of cells in very low densities at suboptimal pH, rather than direct effects of neuraminidase itself.

Tumor regression experiments.—This type of experiment attempts to induce regression of established tumors by administering neuraminidase-treated malignant cells of the same type. Well controlled experiments of this type are exemplified by the work of Simmons and collaborators (*63–65*). Because of the obvious applications of this work to the therapy of solid tumors, it has aroused a great deal of interest.

In one series of experiments (*63*, *64*), syngeneic mice were transplanted with 2 fibrosarcomas, MC–42 and MC–43, originally induced in C3H/HeJ mice with 3-methylcholanthrene; they are specifically immunogenic, since the MC–42 does not immunize animals against the MC–43 tumor, or vice versa, and they do not regress spontaneously (*66*). Animals bearing established tumors were given inocula of tumor cells treated with active or heat-inactivated neuraminidase combined in some cases with BCG. Total regression of some fibrosarcomas followed inoculation of neuraminidase-treated tumor cells. Regression was not induced by inoculation of cells treated with inactivated neuraminidase or by cells incubated with neuraminidase and an excess of free sialic acid. The latter experiment involved competitive inhibition by substrate analogues and indicated that the effects of the enzyme were indeed due to its enzymatic activities. Tumor regression could be induced only by inoculation of neuraminidase-treated cells of identical type to those in the tumor. Thus enzyme-treated MC–42 cells did not cause MC–43 tumors to regress, and vice versa. Against the well-known background of the unique immunologic properties of chemically induced tumors, this observation can be reasonably explained only in terms of immunospecificity. The effects of injection of neuraminidase-treated cells was "magnified" by the simultaneous administration of BCG. The evidence of these authors suggests that, with these two chemically induced tumors, the observed regression was due to the increased immunogenicity of the neuraminidase-treated tumor cells injected subsequently.

Neuraminidase and Increased Immunogenicity

The question of how neuraminidase treatment can make cells more immunogenic and some of the implications will be discussed in the following sections.

Unmasking of antigenic sites on tumor cells by neuraminidase.—This idea was originally advanced by Sanford (*59*) to account for her experimental results in which fewer "takes" were observed in animals given neuraminidase-treated tumor cells than in corresponding controls. Although, as discussed earlier, other unrelated explanations of these observations seem more likely (*62*), the basic concept, that neuraminidase treatment of a cell periphery can expose previously hidden antigenic sites, merits consideration. Masking of other peripheral structures by sialomucins or glycopeptides is feasible, but it must be re-emphasized that neuraminidase will remove only terminal sialic acids from these moieties, and any unmasking effects of treatment with this enzyme must, therefore, be specifically related to removal of sialic acids, not to removal of sialomucins in general. In their studies on TA3 cells, Hauschka et al. (*62*) observed by hemagglutination-inhibition tests that neuraminidase treatment neither inactivated nor unmasked H–2^a and other antigens at TA3 cell surfaces. Sanford and Codington (*67*) also observed that incubation of TA3 cells with this enzyme did not affect the adsorption of H–2, 3, 4, 11, and 28, monospecific antisera.

Other unsuccessful attempts to demonstrate decreased antibody adsorption and/or increased immunolysis after incubation of nucleated cells with neuraminidase have been reported by Currie and Bagshawe (*60*), Ray et al. (*68*) and Lauf et al. (*69*). Schlesinger and Gottesfeld (*70*) observed that neuraminidase treatment of spleen cells increased their sensitivity to the cytotoxic actions of a number of H–2 antisera. However, these experiments were made in the presence of guinea pig serum, which acted as a source of complement, and incubation of the spleen cells with the enzyme resulted in an increase in adsorption of cytotoxic factors in the guinea pig serum. This increase may possibly be related to the reduction in mutual electrostatic repulsion between the cell surfaces and the complement components, which also carry a net negative charge (*71*) after enzyme-induced removal of surface sialic acids. However, work on the effects of neuraminidase on the adsorption of polyanions to tumor cells argues against such a simplistic approach, since great increases in uptake have been demonstrated to be due to increases in the number of dead cells in a population (*72*) rather than to the effects of neuraminidase per se (*73*). Schlesinger and Gottesfeld (*70*) also noted that neuraminidase had no consistent effect on the adsorption of H–2 or Θ-antibodies by lymphoid cells.

If the hypothesis, discussed earlier, of the zonal distribution of neuraminidase-susceptible sialic acids at the cell periphery is correct and is a general phenomenon, then possibly a variety of surface "receptors," including antigenic sites, could be located either close to, or relatively distant from, the sialic acid rich zones. It is, therefore, feasible that the accessibility of antigenic regions of the cell periphery to antibodies could be increased by neuraminidase treatment, if these regions were close to the postulated high-charge density zones. If however, the sites were far away from the zones, relative to the molecular dimensions of the antibodies, neuraminidase treatment would be expected to have little or no effect on this mode of antigenic expression. Many examples of the effects of localized charge on the analogous situation of hapten/antibody interactions are given by Pressman and Grossberg (*58*). This may explain the observations of Grothaus et al. (*74*) that, after incubation with neuraminidase, the susceptibility of human lymphocytes to lysis by antisera directed against HL–A and other antigens was increased, and those of Kassulke et al. (*75*) that neuraminidase treatment increased the amount of A and H antigens detectable at the surfaces of human leukemic leukocytes.

In erythrocytes, neuraminidase treatment apparently exposes ABH (*76*) and Forssmann (*77*) antigens. Although it would be unwise to make the dogmatic statement that neuraminidase *never* affects the accessibility of tumor-specific transplantation antigens at the surfaces of malignant cells to antibodies, such an effect has yet to be demonstrated.

Facilitated contact with killer cells by treatment of target cells with enzyme.—The surfaces of all mammalian cells so far examined carry a net negative charge. Contact between cell surfaces must, therefore, be resisted by the mutual electrostatic repulsion resulting from the interactions of the ionic microenvironments surrounding these charged surfaces. The physical background leading to computation of both electrostatic and electrodynamic contributions to interaction energies between cells has recently been reviewed (*78*); some of the many problems associated with applying the background physics to the biologic situation have also recently been detailed (*79*).

Many electron micrographic data show that initial contacts between cells are made by comparatively small fractions of their total surface areas. For bonds, which have enough total energy to confer adhesive stability, to be formed between these surface regions, these bonds must achieve so-called primary attractive minima, which requires that the involved surfaces be within approximately 0.5 nm of each other. Computations of the interaction energies between regions of cells over a wide range of numerical representations strongly suggest that approach to within 0.5 nm is *initially* impossible because of the magnitude of the potential energy repulsion barriers. Thus cell-surface negative charge dictates that, if high energy bonds are to be formed between regions of cells, then some mechanism must exist for "tunnelling" through these potential energy barriers. Studies on the rates of cell adhesion to protein-coated coverslips in vitro imply that, under specified cultural conditions, such tunnelling mechanisms take tens of minutes (*80*). Tunnelling may involve the production of exudates by the approaching cells which act as "glues" (*81*) by virtue of their charge characteristics. Adhesion of this type may also initially involve the repression of antiadhesion factors (*82*) and/or the sensing of surface regions having lower than average charge density (*48*). Thus, although surface charge appears to be significant in determining that intimate contact and adhesion between cells shall *not* occur, it does not in itself explain adequately how these phenomena do occur.

Computations of total interaction energies between cells suggest that reductions in cell-surface negativity of the order brought about by neuraminidase treatment are unlikely to facilitate cell contact by direct mechanisms, because the residual net negativity is still sufficient for potential energy barriers to prevent contact (*83*). In addition, if the cell-surface sialic acids have a zonal distribution, on energetic grounds contacts will occur in lower-charge density, interzonal regions, and neuraminidase treatment will not affect contact with these areas of the cell surface.

Another possibility is that incubation of cells with neuraminidase exposes positive surface charges which attract negatively charged regions of another cell. Although this exposure of positively charged groups, which were previously undetectable by electrokinetic techniques, has ~~been~~ described in human erythrocytes, it has not been observed in nucleated cells (*84*). From this evidence, neuraminidase treatment would not be expected to affect cell interactions.

After treatment with neuraminidase under optimal conditions, cells tend to replace the enzymatically removed sialic acids within a few hours. Therefore, experiments designed to determine the effects of such treatment should either be shorter than this replacement time, which is not true in mixed lymphocyte reactions for example, or, alternatively, the experiments must be made with amounts of neuraminidase adequate to remove surface sialic acids as they are replaced. In the latter case, particularly in experiments in vitro, it should not be overlooked that the serum in the culture medium, particularly calf serum, usually contains significant amounts of sialoprotein which constitute a source of competitive inhibition for the enzyme.

In short-term experiments, the rate of adhesion of Ehrlich ascites tumor cells to protein-coated glass coverslips for up to 3 hours was not significantly affected by prior incubation with neuraminidase, which caused a 24% reduction in their net surface negativity (*85*). Kolodny (*86*) was also unable to demonstrate an effect of neuraminidase on the attachment of cells to culture dishes. Using neural retina cells from chick embryos cultured with neuraminidase, McQuiddy and Lilien (*87*) could not demonstrate an effect on the size or number of cell aggregates formed for up to 48 hours.

In other in vitro experiments, the adhesion or nonadhesion of two cell lines to glass could not be correlated with either net surface negativity or the contributions of sialic acids to this parameter (*48*). Two unexpected observations were those of Cormack (*88*) who noted that incubation of Walker 256 carcinoma cells with neuraminidase reduced their rate of attachment to mesothelium in vitro as judged by indirect methods, and Kemp (*89*) who observed that the mutual reaggregation of dispersed embryonic chick-muscle cells was inhibited by prior treatment with the enzyme.

The cytotoxic effects of immunized lymphocytes and macrophages on malignant cells appear to involve two steps: 1) a specific adhesion process (*90*) requiring, by analogy with antigen/antibody interactions, the approach to within approximately 0.5 nm of lymphocyte surface receptors and complementary surface antigenic determinants on the malignant target cells; 2) killing the target cells.

The effects of neuraminidase treatment of P815 mastocytoma target cells on their immunolysis by spleen cells from immunized and nonimmunized mice were investigated by Weiss and Cudney (*91*). In a highly sensitive in vitro system in which immunolysis was quantitated by measurements of ^{51}Cr release from labeled P815 cells, neuraminidase treatment of the target cells did not increase susceptibility to attack by sensitized spleen cells. Killing proceeded despite a substantial contribution of sialic acid moieties to the net negative surface charge of the untreated P815 cells, and destruction was not enhanced by enzymatic removal of substantial amounts of bound surface sialic acids, as evidenced by a 60% reduction in (anodic) electrophoretic mobility. At least in this case, no evidence was obtained to support the suggestion that neuraminidase treatment of tumor cells enhances their contact with or destruction by killer cells.

Facilitation of other interactions involving killer and target cells.—Target cells may be killed directly by immune lymphocytes or by macrophages which have been made specifically cytotoxic as a result of a factor produced by lymphocytes originating in the thymus of immune animals (*92*). Feldman and Basten (*93*) recently reported evidence suggesting that sensitized T-cells interact with antigen to produce a factor causing antigen to be concentrated on macrophages which in turn pass on information to B-cells; this leads to the production of humoral antibodies. These and many other communications emphasize the importance of contact between T-cells and antigens as the initial event in an immune response. Other work emphasizes the part played by macrophages in mediating the contact destruction of tumor cells (*94, 95*), in releasing a toxic factor induced by their interactions with target cells (*96*), and in processing antigen (*97*). For example, mitotic activity (i.e., "transformation") of lymphocytes can result from stimulation by macrophages which have previously interacted with antigens (*98*).

When cells are incubated with enzymes, the possibility always exists that the enzymes may be adsorbed to the cell surface (*141*). The surface adsorption of trypsin, which has a net positive charge, partially accounts for the loss in net surface negativity of embryonic cells after incubation with this enzyme (*99*); and Rosenberg (*100*) has shown that trypsin taken up by cells may be slowly released in an active form which degrades protein at their environmental interphase. Neuraminidase labeled with a fluorescent dye, and in an active form, adsorbed to cell surfaces (*101*), but whether any remains after washing is an open question because of the low sensitivity of the technique. Thus it would be of interest to examine the surfaces of neuraminidase-treated cells for residual enzyme after exposure to fluorescent and/or isotopically labeled antibodies prepared against neuraminidase. A strong suggestion that neuraminidase is adsorbed to cell surfaces, particularly after exposure to high concentrations, also comes from electrokinetic studies on human erythrocytes incubated with various concentrations of then enzyme (*102*), after which their electrophoretic mobilities indicate a progressive loss of net surface negativity over the enzyme concentration range of 0.001–10 U/10^6 cells. After exposure to the higher concentrations, the erythrocytes migrate too slowly in an electric field to permit accurate measurement. In parallel with these electrokinetic studies, chemical assays for free sialic acids revealed no increase after incubation with neuraminidase in concentrations higher than 0.01 U/10^6 cells.

Since studies on the release of ^{51}Cr-labeled materials from erythrocytes incubated with concentrations of neuraminidase up to 200 U/10^6 cells revealed no difference from controls, it seems unlikely that the adsorption of leaked materials to the erythrocyte surfaces could account for the changes in their electrophoretic mobilities as described in other situations (*103*). Similar changes in the surfaces of inert inorganic particles have been described after their incubation with neuraminidase which at physiologic pH carries a net positive charge. Such changes occured with final concentrations of 250 U of enzyme, but were not demonstrable in the presence of 5 U (*104*). Thus the progressive decrease in surface negativity described earlier in erythrocytes is due probably to adsorption of neuraminidase to the surfaces. In many of their experiments, Simmons and colleagues used neuraminidase in final concentrations of 200 U/10^6 cells as opposed to more usual levels of 1 U/10^6 cells. Although such high doses of enzyme are not in themselves immediately cytotoxic, the possibility exists, particularly with high doses but also with more usual doses, that some enzyme may remain adsorbed to the cell surface and/or be released subsequently, thereby modifying the surfaces of nearby lymphocytes and macrophages and hence their relevant interactions.

In addition to the exogenous neuraminidase added by the experimentalist, endogenous enzyme should not be overlooked. Lysosome-bound neuraminidase is present in different cells, including those from rat mammary glands (*105*), rat liver and kidney (*106*), and Ehrlich ascites tumor cells (*107*). Lysosomes may be activated by a wide range of stimuli, including antisera (*108*, *109*), and liberated enzymes may modify the surfaces of the cells releasing them by an autolytic process (*110*) that may alter certain cell-contact reactions (*111*). Lysosomal enzymes may also attack the surfaces of adjacent cells which are not themselves the source of the enzymes (*112*).

In the following section, some aspects of the effects of neuraminidase on both killer and target cells will be considered.

In mixed lymphocyte cultures, Lundgren and Simmons (*113*) show that treatment of X-irradiated target cells with neuraminidase enhanced their stimulatory activity as assessed by increased incorporation of tritiated thymidine into the responding cells. However, no difference was observed between incorporation in responding cells treated or not treated with neuraminidase when these were cultured with target cells which had *not* been exposed to the enzyme. These results, therefore, suggested that the effect of neuraminidase in interactions between target and responding cells, was limited to its effect on the target cells.

However, in their own in vitro studies, Weiss and Cudney (*91*) showed that in mastocytoma/sensitized spleen cell interactions where neuraminidase treatment of the target cells had no effect on immunolysis, enzyme treatment of the spleen cells produced significant enhancement in killing. These results were contrary to those of Lundgren and Simmons (*113*) in suggesting that in their in vitro system at least, the effects of neuraminidase are mediated through the killer cells. Support for Weiss and Cudney's interpretation comes from the work of Tin Han (*114*) on human mixed "lymphocyte" cultures that contain all types of peripheral leukocytes. In these experiments, prior incubation of the target population with neuraminidase produced an increased rate of incorporation of isotope in the responding population not treated by the enzyme. However, in contrast to Lundgren and Simmons (*113*), Tin Han observed that treatment of the responding cell population with neuraminidase resulted in increased incorporation when the cells interacted with target populations treated or not treated with the enzyme. Thus once again emphasis is focused on the effects of neuraminidase on the responding cells rather than their cellular targets.

Much experimental evidence indicates that incubation of lymphoid cells with neuraminidase alters their surfaces. For example, electrokinetic studies on neuraminidase-treated cells reveal a significant loss of surface negativity of approximately 40% in freshly isolated, human peripheral lymphocytes (*115*); 25–28% in mouse lymph node cells (*116*); 36–54% in "normal" lymphoid cells maintained in vitro (*32*), and 40–50% in circulating blast cells from patients with acute and chronic lymphocytic leukemia (*32*). Human lymphocytes treated with neuraminidase are also more susceptible to lysis by antisera directed against HL–A, Θ, and TL antibodies than corresponding controls (*74*); and sensitized human lymphocytes incubated with the enzyme have increased sensitivity to concanavalin A, pokeweed mitogens, and various other soluble antigens used for skin testing (*117*).

The peripheral properties of macrophages are demonstrably changed by incubation with neuraminidase. In human circulating monocytes prepared by polyvinylpyrrolidone treatment of whole blood, a 53% reduction in surface charge density was demonstrated by measurements of cell electrophoretic mobility following enzyme treatment (*118*). Human monocytes obtained from blood by a different technique had their mobilities reduced by approximately 19%, and macrophages from peritoneal exudates of mice also exhibited mean reductions of approximately 19% after incubation with neuraminidase (*115*). Associated with this loss of surface negativity in human monocytes is an increase in their deformability (*118*).

Murine Sarcoma 37 and Ehrlich ascites tumor cells also show an increased deformability associated with loss of surface negativity after neuraminidase treatment (*119*). Such deformability can be demonstrated by a reduction in the negative pressure required to suck a hemispherical bulge from individual cells into micropipettes having an internal diameter of 5 μm. That the rigidity of the cell periphery is partially maintained by the mutual repulsion between the ionized carboxyl groups of sialic acids and that their removal would make this structure more deformable were suggested by Gottschalk's (*120*) studies of the physical properties of ovine submaxillary gland sialoproteins in which electrostatic repulsion between their acid prosthetic groups confers structural rigidity on the underlying protein core. Increased cell deformability is not a unique effect of neuraminidase, since it may also be brought about by factors breaking various types of bonds in other structural components of the cell periphery, including proteolytic enzymes (*121*) and removal of calcium ions (*122*). The increased flexibility is not inevitably the consequence of reduced cell surface charge, since ribonuclease which also reduces charge produced no detectable change in deformability (*123*). The question arises whether neuraminidase-induced changes in either target cells or macrophages can account for the enhanced immunogenicity of the former.

Interactions between tumor cells and macrophages often result in destruction and/or phagocytosis of the former (*124*, *125*). Electron micrographs of interacting target cells and immune macrophages reveal a close apposition between the cells along extremely convoluted, interdigitated margins (*126*, *127*). At areas of close contact adherent parts of the target cell are apparently pulled into the macrophage and pinched off. The fate of the ingested portions and the remaining uningested parts is not known, nor is it known whether this type of interaction is concerned with antigen processing or is in some way unique to cell-associated immune reactions. Whatever their functional significance, engulfing interactions of this type could be facilitated by increasing the deformability of the target cells and/or the macrophages. Thus it is interesting that neuraminidase treatment of macrophages, which increases their deformability, also increases their ability to phagocytose negatively charged particles in vitro (*118*).

The degree of ingestion of opsonized erythrocytes of mouse peritoneal macrophages is greatly increased if the erythrocytes are treated with neuraminidase before opsonization (*128*). Unpublished work of Horwitz and Weiss [cited in (*119*)] showed suggestive increases in the deformability of erythrocytes incubated with neuraminidase. In a critical review of phagocytosis, North (*129*) comments that, apart from the fact that phagocytosis is influenced by serum opsonins and depends on ATP, practically nothing else is known about the process. After incubation of mouse macrophages with neuraminidase, the adherence of opsonized bacteria to them is increased (*131*). By facilitating the adsorption of cytophilic antibodies and/or opsonins to target cells and/or macrophages, neuraminidase may enhance primarily the efferent limb, i.e., the host-cell mediated part of the immune response.

Indirect effects of neuraminidase.—There is no question that neuraminidase can by its direct actions change the physicochemical properties of the peripheries of many different types of cells. However, neuraminidase can enter and digest internal organelles without destroying the cells (*101*). In addition to modifying the surface properties mentioned earlier, incubation with neuraminidase can also affect potassium transport (*130*) by reducing its unidirectional flux across tumor cell membranes (*132*). The reversible cation exchange behavior of sialoglycopeptides from human erythrocytes has been demonstrated (*133*). Neuraminidase treatment of HeLa cells decreases their net accumulation of amino acid without altering its efflux from previously loaded cells, which suggests that sialic acids in the cell periphery may be significant in amino acid transport (*134*). An important indication of the function of sialic acid residues at the cell surface in relation to growth control is the observation that low concentrations of neuraminidase initiate proliferation in stationary cell cultures and cause an early increase in sugar uptake (*135*). Therefore, even when seemingly adequate explanations account for the effects of neuraminidase exclusively in terms of its direct effects on the cell periphery, such peripheral effects may be of secondary importance in the present context, compared with subsequent intracellular events which may also be manifested as changes in cell surface regions.

Is Neuraminidase Really Necessary?

The absence of tumors or the presence of small, slowly growing tumors in animals receiving neuraminidase-treated tumor cells may be accounted for satisfactorily by the explanation that less than LD100 doses of undamaged, viable cells were given. The regression of such inocula could be accelerated by the simultaneous administration of a large number of nonviable cells retaining, in chemically induced tumors, the specific antigenicity characteristic of neoplasms of this type. It would be expected that similar results could be obtained by giving animals inocula containing similar proportions of living and dead cells not treated with neuraminidase. This has, in fact, been described with TA3 cells (*62*), and comparisons of the lethality of inoculated BW10232 mouse mammary carcinoma cells fail to reveal a significant difference between X-irradiated cells and those incubated with neuraminidase after exposure to 5000 R (*136*).

In experiments in which regression of established tumors is brought about by inoculation with neuraminidase-treated neoplastic cells, with or without the simultaneous administration of BCG, it is presently impossible to give a dogmatic explanation of the results. If a key part of the enhanced immunologic response of the host to tumors is the facilitation of phagocytosis of the tumor cells by macrophages, then any factor promoting phagocytosis would serve the purpose. Making cancer cells or macrophages more deformable by any agent without destroying their relevant antigenic markers would be expected to enhance the response. Although proteolytic enzymes make cells more flexible, they cause the release of antigenic glycopeptides from cells (*34*, *137*), the removal of which would alter other parameters. Therefore, experiments involving challenge doses of cells treated with these enzymes may well be inconclusive. Cells also become more deformable when degenerative changes are occurring in them, and lysosomal and other enzymes bring about autolysis. Thus lethally X-irradiated cells might also exhibit immunogenic enhancement equal to that shown by those treated with neuraminidase. Comparisons of this nature should be made, since X-irradiated vaccines of tumor cells sometimes (*138*, *139*), but not invariably (*140*), show enhanced immunogenic activity when their tumor transplantation antigens are neither unmasked nor denatured but are apparently expressed in normal titers.

Phagocytosis can also be enhanced by increased adsorption of opsonins or opsonin-like materials to the surfaces of tumor cells and/or macrophages. It would be of interest, therefore, to determine the effects of adsorption-promoting agents other than neuraminidase on the immune response.

Since many experiments on the effects of neuraminidase-treated cells on tumor regression have been made on only a few types of transplanted tumors, it would be extremely unwise, at present, to draw any far-reaching conclusions on the applicability of this approach to the immunotherapy of autochthonous cancer in general.

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ADDENDUM

This Editorial was written in response to a number of papers which showed that treatment of animals with various neuraminidase-based modalities favorably affected some aspects of their response to transplanted cancers. Although these observations were not questioned, their unique interpretation in the "unmasking" of tumor-specific cell surface antigens, which then provoked an enhanced immune response, was usually assumed, rather than unequivocally demonstrated. One of my main thrusts was to point out that neuraminidase affected a number of other cellular parameters in addition to unmasking and that it would be prudent to clarify the issue by experimentation rather than polemics before subjecting patients to the nebulous entity of immunotherapy. Some of these reservations have now been expressed by others (*1*).

Since publication, it has been demonstrated yet again that, although 1) the amounts of neuraminidase-sensitive sialic acids present at the cell periphery bear no constant or general relationship to cancer (*2*) and 2) sialic acids may "mask" some antigenic determinant sites, they are also part of the determinant sites of other antigens (*3*). Therefore, neuraminidase could cause functional gain or loss in immunogenicity. In many of the early experiments on the effects of injection of neuraminidase-treated cancer cells on tumor regression, enormous doses of the enzyme were used. I suggested that neuraminidase was also administered adsorbed to the cancer cells. Sedlacek et al. (*1*) substantiate this view and, in addition, note that neuraminidase can exhibit adjuvant activity. LaMont and Isselbacher (*4*) have shown that neuraminidase-enhanced, lectin-induced agglutination of erythrocytes, which is an example of a cell contact phenomenon, depends on mechanisms other than removal of sialic acid.

However, the issue of highest priority is whether we have a technique for improving the treatment of patients with cancer, not whether we can offer explanations for its success. Sedlacek et al. (*5*) studied the response of spontaneous mammary tumors in dogs to injections of autologous mitamycin-killed cells, with or without treatment with neuraminidase. They observed that when dogs were given injections of 2×10^7 mitamycin-treated cells at sites distant from their tumors, the cancer progressed in 8 of 10 animals. When the animals were given 2×10^7 cells treated with mitamycin and neuraminidase, tumor regression was observed in 13 of 15 dogs; however, when 10^8 cells treated with both agents were administered, the tumor progressed in 8 of 8 animals. The local response to neuraminidase-treated cells is similar to that seen after BCG administration (*6*). One is reminded that, in some tumor-bearing animals, injections of BCG may cause enhancement of tumor growth in the clinical situation (*7*) and the immune response may promote some parts of the metastatic process (*8*). At present, I am aware of no convincing evidence that treatment of human cancer with neuraminidase-treated cells is any more effective than any other form of "immunotherapy," which is of unproved benefit in patients with established cancer. In this regard, Sansing and Kollmorgen's detailed study (*9*) of the humoral response in mice to neuraminidase-treated L1210 leukemia cells shows that tumor remission did not occur when the tumor burden had reached 10^7 cells. The attempts of Rosato et al. (*10*) to treat patients with cancer with injections of autochthonous, neuraminidase-treated cancer cells are clinically inconclusive. Perhaps more important than the likelihood of not doing good is the indication that under some circumstances, administration of neuraminidase-treated cells may do harm.

As far as I am aware, administration of neuraminidase-treated cells to patients in an attempt to stimulate their immune response against their cancers has not proved effective. Its use as a therapeutic modality is unlikely.

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GUEST EDITORIAL——by **Aaron E. Freeman and Robert J. Huebner**
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Problems in Interpretation of Experimental Evidence of Cell Transformation

IN VITRO TRANSFORMATION has been used to characterize the oncogenic activity of many viruses and chemicals, and we support these claims when the experiments are properly controlled and evaluated. However, in too many papers there is no evidence of actual transformation, or if it had occurred, that it was actually due to the agent presumed to "cause" the changes observed. Frequently it is obvious that neither the authors, reviewers, nor editors had considered alternate possibilities. We think that, in at least some instances, the observed morphologic changes were artifactual or overinterpreted. We list here some problems that should be considered and suggest some steps which can provide reasonable evidence that transformation has occurred and was actually due to the specific agents used.

What is Transformation?

The first basic problem in all studies of transformation is its definition. The Tissue Culture Association in its "Proposed Usage of Animal Tissue Culture Terms—Fifth Draft" suggested the following definition:

> "Cultural alteration. This term is used to indicate a persistent change in the properties and behavior of a culture, e.g., altered morphology, chromosome constitution, virus susceptibility, nutritional requirements, proliferative capacity, malignant characteristics, etc. The term should always be qualified by a precise description of the change which has occurred in the culture. The term cell transformation should be reserved to mean changes induced in the cells by the introduction of new genetic material. The nature and source of the genetic material inducing the change should be specified."

Since, in actual practice, transformation means many things to many people, it is imperative for each journal to insist that the authors carefully state their use of the term. Later we will discuss in detail our suggestions regarding definitions of transformation. For the present, however, we consider transformation to be the acquisition of a series of abnormal genotypic characteristics in a cell culture that exhibits normal characteristics under controlled conditions. But what are these abnormal genotypic changes characterizing the transformed culture? This question leads directly to the next problem.

Are There Adequate Criteria to Show That Transformation Has Occurred?

The most commonly used nonspecific markers of transformation are changes in morphology (cell type, colony type, loss of contact inhibition, loss

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

of cell orientation, increased number of nucleoli, changes in nuclear-cytoplasmic ratio), increased saturation density, chromosomal aberration, loss of anchorage dependence, "immortality" in culture, and ability to produce tumors in animals. Few transformed cells possess all these characteristics. Further, there is no absolute correlation between any single marker and the ability of cells to grow in animals as transplantable, malignant tumors. Presence or lack of any single marker does not prove or rule out transformation of cells. Tumorigenicity is a strong marker of transformation; however, some cultures such as adenovirus type 2-transformed rat embryo cells, which fulfill the Tissue Culture Association definition of transformation, are not tumorigenic. In addition, certain cultures of normal cells may grow as transplantable tumors in hamsters or mice whose immune responses have been impaired by irradiation, drugs, or antilymphocyte serum. Further, tumorigenicity of human cells is difficult to demonstrate. In this instance, immortality in culture may be used as strong evidence of transformation. Immortality, however, is of little value as a marker for the transformation of hamster, mouse, or rat cells.

In addition to the nonspecific characteristic of transformed cells, those working with DNA virus-induced cell transformation have a number of markers that specifically implicate the viral genome as the transforming agent. Thus tumor or T antigens can be demonstrated by complement fixation or fluorescent antibody staining. Sometimes specific viral mRNA can also be demonstrated. In sarcoma virus-induced transformation, the entire virus genome may be rescued by a helper virus from a nonproducer cell culture, and the resultant pseudotype virus can be shown to be tumorigenic in the appropriate host. With the use of specific markers and some previously mentioned nonspecific ones, many excellent papers have been written on the transformation of cell cultures by DNA [polyoma, simian virus 40 (SV40), adenovirus] and RNA (Rous sarcoma, mouse sarcoma, cat sarcoma) viruses.

A more difficult problem is encountered in studies of spontaneous transformation or transformation induced by chemical carcinogens and/or the C-type RNA leukemia viruses, because there are no specific markers of transformation for these cultures. In these instances, the case for transformation must be built on the accumulation of a number of abnormal nonspecific characteristics clearly defined and critically studied. However, too many papers are based entirely on the production of "transformed foci." While these papers may be valid, verifying tests are needed. Pseudofoci may be caused by a number of factors—presence of flocculent material in the serum, toxic areas on the culture vessel, and piling or retraction of the culture associated with aging. If transformation has occurred, the observed changes should be hereditary. Thus on further subculture transformed foci should yield transformed cell lines, and the progeny cultures should possess the same or enhanced characteristics on which the transformation is based.

Are Morphologic Changes Related to Infection and Not to Transformation?

In some instances carrier cultures can be established, and the chronically infected cell may differ morphologically from uninfected cells. In other cases the virus may produce a slow lytic effect. In the short run, foci of altered cells appear, but represent dying rather than transformed cells. With this type of "transformation," we can expect the culture to die after every cell becomes transformed. Culture death is usually a good indication that the cells are not transformed, since most cells (except chicken cells) of transformed cultures will be "immortal." The cells should be monitored for infectious virus. Lack of infectious virus and demonstration of specific viral genome expression can provide good evidence of transformation. Of course, cells carrying virus may also be transformed, but here it should be shown that the newly acquired cultural characteristics are independent of the viral infection. When virus is present, the cells may be grown in specific antisera, to show that the induced changes are independent of the infection.

As a case in point, in our laboratory we once observed an adenovirus type-12 (AV-12)-infected human embryonic lung, which developed morphologically altered cells appearing as foci of epithelioid cells on a fibroblastic background.

Gradually these altered cells became more predominant. Serologic studies demonstrated that the foci were producing AV oncogenic group A tumor antigens and AV-12 viral antigens. Cultures not grown in specific AV-12 antiserum gradually became more altered and died; cultures grown in the specific antiserum returned to normal. Thus we were able to show that the observed morphologic changes were due to infection and not transformation. Consequently, it was apparent that we had not transformed human cells with AV-12.

A similar problem may be encountered by workers in chemical carcinogenesis. Just as viral infection may cause morphologic alterations, so may chemical treatment. It is not enough to demonstrate that the morphologic changes persist for a short time after removal of the chemical, since very small quantities of some chemicals produce effects that last for several subdivisions in a "chemical-free" medium. Here again, the best evidence is the development of a continuous cell line with stable hereditary characteristics.

What is a Cell-Free Extract?

In our experience, many artifactual observations have been caused by the assumption that the virus preparations were cell free. We know this has also happened to others. Supernatants often contain viable cells; furthermore, freezing and thawing or sonic disruption does not kill all cells. Even filtration does not assure a cell-free preparation, because the filters may leak. One or a few foreign cells, added with the virus inoculum, may appear in due time as "transformed foci."

We suggest that cell-free extracts be prepared by filtration (we use a 0.22-μ pore). After the virus has been filtered, the integrity of the same filter should be tested with an appropriate bacterial culture. If bacteria cross the filter, it is not safe to assume that the filtrate is cell free. In addition to the filter tests, specific characteristics of the cell lines can indicate that the transformed cells were derived from the normal parent culture. The cell line used to grow a transforming virus should be characterized by specific markers such as species, sex, and marker chromosomes, or by a specific characteristic such as interferon sensitivity. One or more of these markers should also be clearly different for the substrate cell. The transformed cells should be examined to make sure they are derived from the substrate cells. When the virus is grown in one species of cell and the substrate cell is another species, a simple chromosomal evaluation may indicate whether cell contamination is involved. When the cell used to produce the seed virus is the same species as the substrate cell used for the transformation study, at the very least, the virus should be grown in a diploid cell characterized as male or female; the substrate cell should be of the opposite sex.

Is the "Transformation" Caused by Some Contaminating Agent?

A variety of normal and malignant cultures is usually carried in most cell culture laboratories. Many common laboratory errors can confuse or mix cultures. One transformed culture fed from the same vessel as the experimental normal culture can lead to transfer of a few cells that will appear in time as transformed foci. Frank contamination of this type has been reported many times. However, the cell characterization studies mentioned under the previous problem regarding cell-free extracts will also help to prove that cell contamination did not enter from any source.

Contamination by bacteria and mycoplasma may also interfere with a study on transformation. Mycoplasma, in particular, should be ruled out, since some investigators feel that these agents have a pseudotransformation potential of their own. This type of contamination offers no real problem, since the bacteriologic tests for these agents are readily available. Contaminants with other oncogenic agents, however, present a more difficult problem. For rodent cells, mouse antibody-production tests can identify the known rodent viruses, including polyoma. Specific immunologic studies can be used to rule out known tumor virus such as SV40. The purity of the virus preparation can be improved by gradient separation or by means of a limiting endpoint dilution procedure. Electron microscopy can be used to screen for unexpected particle types. Despite all these precautions, however, we must always be aware that viruses do not always exist as infectious agents, that our substrate cell line may harbor an endogenous viral genome, and that these latent viruses may be ultimately implicated as the causative agents of transformation.

Are Investigators Biased?

The problem of bias in research cannot be overlooked. In transformation experiments, the technician may treat or read the cultures a little differently and thus, in a subtle manner, force the experiment to a predesired ending. The senior investigator may select data which fit, and reject data which do not fit, his hypothesis. These normal investigatory practices do not represent dishonesty, but are examples of bias, difficult for even the most intrepid researcher to avoid. Thus after the basic experimental design has been established, coded samples, preferably keyed by an outside source, should be tested. If the original observations are repeatable, coded samples should offer no problems. On the other hand, if the blind studies do not behave as expected, the original investigator should be the first to know.

Discussion

A typical paper on cell transformation might present the following story. A cell line with a normally low frequency of spontaneous transformation was treated with a cell-free preparation of a certain virus which had never before been shown to be oncogenic. A novel treatment was used. In due time transformed foci appeared in the infected cultures, but not in the normal controls. From the foci, cell lines were derived that were not only morphologically different from the original cell culture but also produced tumors when infected subcutaneously into newborn isologous animals. The experiment was repeated several times with similar results. The author concluded that virus "X" was carcinogenic. We suggest alternate explanations.

Over the years, we have been involved in many studies on transformation, both as senior investigators and as advisors for other workers who requested us to monitor their cultures. Under these circumstances, we have observed several cultures attributed to be transformed, but which were actually artifacts. In one instance we showed that a mouse cell line reported to be transformed by human virus was, in fact, a human cell line derived from the inoculum. In another, we found that a transformed human cell line transplantable in hamsters was actually an AV-12/SV40-transformed hamster cell line. In still another, we found that a chemically induced transformation was really due to a polyoma virus contamination.

Unless each author, editor, and reviewer considers transformation in terms of critical standards, an unfortunate house of cards can be built and sometimes sold to others. When new oncogenic agents are involved and reagents are unavailable, it is extremely difficult to demonstrate that the reported causative agent is responsible for the observed morphologic changes. In these instances, it is even more important that the authors consider and discuss how they have confirmed their findings.

Earlier we stated our definition of transformation as the expression of a series of abnormal genotypic characteristics in a cell culture that exhibits normal characteristics under controlled conditions. By this definition, changes not stable and hereditary, or genotypic changes from one normal state to another, would be considered cultural alterations. We prefer the term "genotype transformation" for cultures that have acquired abnormal genotypic characteristics, but that cannot be shown to produce tumors in animals. Finally, we prefer the term "malignant transformation" for cultures not only genotypically transformed, but tumorigenic as well.

We have deliberately omitted specific references, because it is not our intention to question the validity of any single published paper. Our purpose is to point out that many investigators have failed to consider the problems in interpretation of experimental evidence of a transformation.

ADDENDUM

Certain chemical agents tested in vivo may elicit specific responses depending upon the species, sex, age, route of inoculation, and target organ of the test animals. Most of these variations can be explained by qualitative and/or quantitative differences in the metabolic pathways by which the chemical is detoxified and eliminated.

When chemical agents are tested for transforming activity in vitro, we must ask whether the cell population-at-risk is representative of one or both of the following cell types: 1) those cells which become tumors in vitro, and 2) those cells which metabolize distal carcinogenic agents to proximal forms.

Unless both cell types are present in the test cultures it is improbable that transformation will occur.

Those of us who have attempted to transform human fibroblasts with chemical carcinogens know that this is difficult to do, but some success has been attained, especially with proximal carcinogens. Of course, the human fibroblast in vivo is a rare target cell, as most cancers are of epithelial cell origin. Furthermore, the human fibroblast in vitro has little hydrocarbon-metabolizing activity. Thus we might expect that human fibroblasts in vitro would be resistant to many agents actually carcinogenic in man.

On the other hand, transforming activity in rodent cells may not be relevant to cancer in man. Thus it is highly desirable to conduct transforming experiments in human cells. Two alternative methods may help resolve these problems. The first would be to activate metabolically the chemical to be tested with human liver microsomes. This procedure could fail due to a short half-life of the active intermediate. The second method would be to use metabolically active human epithelial cells derived from specific target organs, i.e., liver, lung, pancreas, skin; however, to date it has been difficult to culture metabolically functional cells representative of these organs.

GUEST EDITORIAL——————————**by Isadore Brodsky, M.D.**

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JNCI 51:329–335, 1973

Role of the Megakaryocyte and Platelet in the Leukemic Process in Mice and Men—A Review and Hypothesis [1]

THE MEGAKARYOCYTE is of major significance in the virus-induced murine and feline leukemias. Electron microscopy revealed that the C-type virus particles of murine and feline leukemias are characteristically in the cytoplasm of megakaryocytes and platelets (*1–3*). In mice, thrombocytopenia can be used in dose-response curves to titer both Friend and Rauscher viruses (*4*, *5*). Text-figure 1 presents a dose-response curve for Rauscher virus. There is a straight-line relationship between virus dilution and degree of thrombocytopenia in both normal and splenectomized mice. The thrombocytopenia occurring after Rauscher virus infection is biphasic (text-fig. 2). The initial dose-dependent thrombocytopenia develops within 48 hours of infection and reaches its nadir 2 weeks later. At 2 weeks the bone marrow is hypercellular with megakaryocytic hyperplasia and there is no infiltration with leukemic cells. Within 4–6 weeks in the surviving mice, recovery is spontaneous. However, 10–24 weeks after infection thrombocytopenia again develops, but is now associated with lymphocytosis in the peripheral blood and leukemic cell infiltration of the bone marrow (*5*).

We have characterized the initial phase of infection with Rauscher virus as a preleukemic state (*5*). Actually, RV probably infects marrow stem cells, since the preleukemic phase is characterized not only by a dose-dependent thrombocytopenia but also by a dose-dependent splenomegaly granulocytosis, monocytosis, and hemolytic anemia (*5*, *7*). Mice given high-titered virus injections frequently do not survive this preleukemic phase, and death is due to a combination of splenic rupture, hemorrhage secondary to the thrombocytopenia, and severe anemia secondary to both hemorrhage and hemolysis. Platelet survival studies performed with selenomethionine (^{75}Se) during the preleukemic phase demonstrate a sharp decrease in platelet survival (*6*). This is most marked in mice previously splenectomized (text-fig. 3). This initial thrombocytopenia is not due to disseminated intravascular coagulation, in that our studies have not demonstrated any significant changes in the coagulation factors—in particular, the fibrinogen concentration and the thrombin-fibrinogen interaction.

Metabolic studies indicate that the initial thrombocytopenia is due to a direct effect of the virus on platelet metabolism (*8*). The nature of

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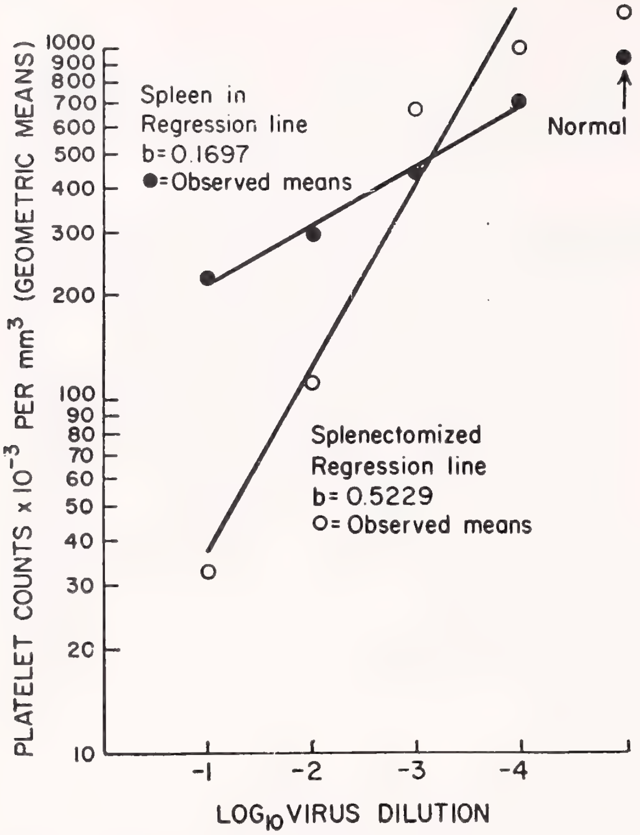
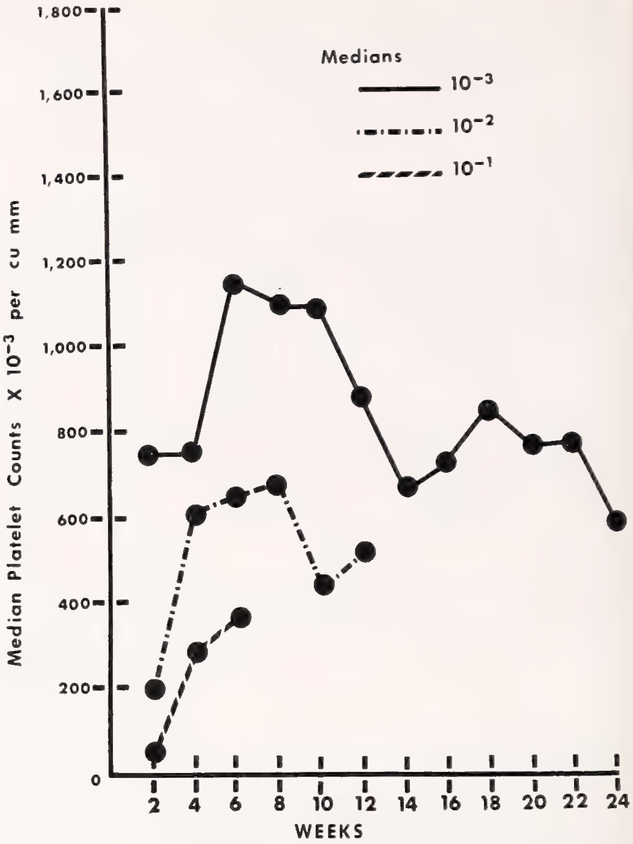
Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.



TEXT-FIGURE 1.—Effect of Rauscher virus on platelet counts in splenectomized and nonsplenectomized mice. Reprinted, with permission, from *Journal of the National Cancer Institute* (*5*).



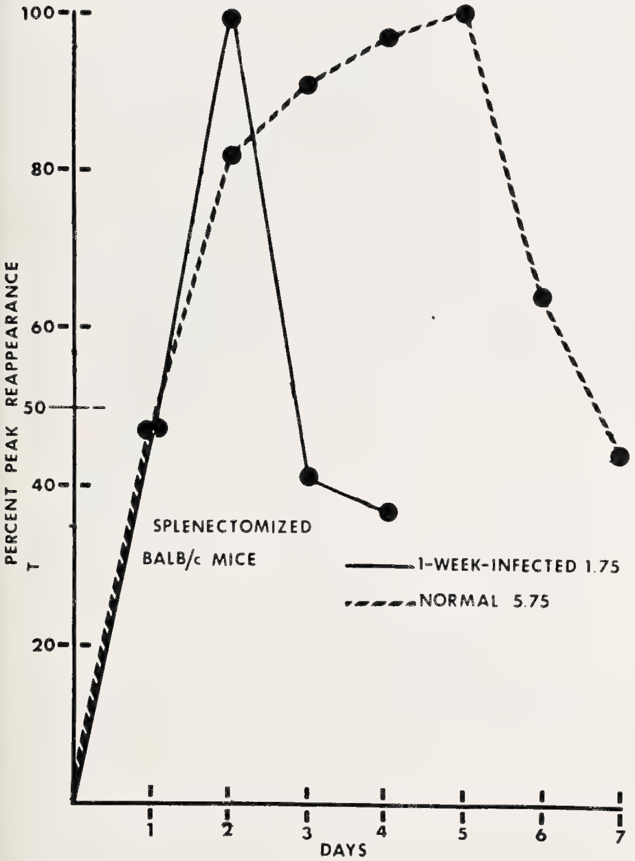
TEXT-FIGURE 2.—Effect of Rauscher virus dilution and duration of infection on the platelet count in splenectomized BALB/c mice. Mean platelet count in uninfected splenectomized BALB/c controls is 1,285,000/mm^3. Reprinted, with permission, from *Cancer Research* (*6*).

the leukemia following Rauscher virus infection remains debatable. Rauscher (*9*) considered it a lymphatic leukemia and our studies support this concept (*5–7*). Other investigators consider the disease to be a form of erythroleukemia (*10, 11*). The effects of Friend virus are similar to those of Rauscher virus, but one Friend virus strain in the preleukemic phase causes a polycythemia and not anemia (*4, 12–14*).

Since both Friend and Rauscher leukemia are caused by inoculation of virus into susceptible mice, the question arises as to whether a preleukemic state, particularly in regard to thrombocytopenia, can be demonstrated in spontaneous AKR murine leukemia. This is indeed the case. AKR mice with an 80–90% incidence of spontaneous lymphatic leukemia by the age of 1 year have a relative thrombocytopenia within 3 weeks of birth, compared to BALB/c mice with a low incidence of spontaneous leukemia. As the AKR mice mature, however, the platelet count increases and then decreases with the onset of leukemia (*15*). The pattern is like that described after infection of mice with high dilutions (10^{-3}) of Rauscher virus (text-fig. 3). The preleukemic platelets of AKR mice are metabolically abnormal as compared to low-leukemia-strain controls (*16*).

Do any analogies exist between murine viral leukemias and leukemia in human beings? Preleukemic states are being described increasingly in human beings and the central role of the megakaryocyte and platelet is being recognized. In 1953 Block et al. called attention to a preleukemic state in humans (*17*). The most consistent finding in their series was a maturation arrest and aplasia of neutrophil precursors in the marrow and neutropenia. However, this abnormal granulopoiesis was frequently associated with alteration in marrow erythroblasts and megakaryocytes.

In these patients, acute granulocytic leukemia ultimately developed after a period varying from several months to 6 years (*17*, *18*). Degos et al. (*19*) reported 3 patients in whom thrombocytopenia was the major preleukemic manifestation. The pattern of thrombocytopenia was similar to that described in Rauscher virus and spontaneous AKR leukemia. The patients recovered from an initial thrombocytopenia with megakaryocytic hyperplasia, only to again develop thrombocytopenia with the onset of the acute leukemic process. One patient studied in the preleukemic phase demonstrated a sharp decrease in platelet survival. In a 13-year-old, Queisser et al. (*20*) demonstrated a distinct abnormality in megakaryocytopoiesis in the preleukemic phase of the disease. They postulated that in this phase a basic proliferation defect of acute leumemia is becoming apparent in the so-called nonleukemic cell system that results in peripheral monocytopenia or pancytopenia.



TEXT-FIGURE 3.—Effect of 10° Rauscher virus infection on platelet survival in splenectomized BALB/c mice. Platelets were labeled with ^{75}Se 7 days after infection. Each *point* represents reappearance of ^{75}Se in the platelets obtained from pooled blood of 3 mice. Results compared to normal uninfected controls. Reprinted, with permission, from *Cancer Research* (*6*).

The AKR mouse probably represents our best experimental model for the human lymphoproliferative disorders (*21*). The pattern of thrombocytopenia in the AKR mouse is like that described in humans with chronic lymphocytic leukemia and chronic lymphosarcoma cell leukemia. In a series of 469 cases reported by Zacharski and Linman (*22*), only 50% of the patients had varying degrees of thrombocytopenia at diagnosis. A significantly greater death rate ($P<0.01$) was noted among patients with moderate-to-marked thrombocytopenia than in those with normal or slightly decreased platelet counts. In the AKR mouse, the second phase of thrombocytopenia is also associated with the onset of leukemia and shortened survival (*15*).

Finally, many human viruses can cause severe thrombocytopenia (*5*). Although such factors as disseminated intravascular coagulation and immune responses must be considered, in most cases thrombocytopenia probably will eventually prove to be due to a direct metabolic effect of the virus on the megakaryocyte and platelet (*5*, *23*). Could a thrombocytopenia-inducing virus such as rubella be potentially leukemogenic in individuals with the appropriate genetic predisposition for the development of leukemia? The rubella virus could even act indirectly by activating "switched off" type C viral oncogenes in the megakaryocytes (*24*, *25*). It might be important to initiate epidemiologic studies, particularly in children, vis-à-vis the acute and late hematologic sequelae of infection with the common childhood viruses. For example, idiopathic thrombocytopenic purpura (ITP), particularly in children, is probably triggered by a viral infection. ITP is characterized by megakaryocytic hyperplasia, with a sharp decrease in platelet survival. The pattern is like that noted in the preleukemic phase of Rauscher virus-infected mice (*23*).

A major difficulty in study of the preleukemic phase in human beings is the lack of precision with which this diagnosis can be made. Ultimately, as in the AKR mouse, one must wait for the development of the full-blown leukemic state. Obviously such cases in man can be based only on fortuituos observations. It is even conceivable that most preleukemic states are entirely reversible. Infectious mononucleosis, a disease most likely caused by a herpesvirus, might well fit into such a category. With these limitations in mind, we have embarked on a study of platelet kinetics in the chronic myeloproliferative disorders: polycythemia vera (PV), myelofibrosis and myeloid metaplasia (MF), and chronic myelocytic leukemia (CML).

PV is a preneoplastic state characterized by a panmyelosis with increased erythropoiesis, granulopoiesis, and megakaryocytopoiesis (*26*). The disease will frequently evolve into MF and in approximately 10–15% of the cases terminate in acute leukemia. A major morphologic characteristic of the bone marrow is an intense megakaryocyte hyperplasia. This human disease bears a superficial resemblance to the disease induced by the Friend virus polycythemia strain (*14*), at least from the standpoint of the development of splenomegaly and bone marrow morphology. A major difference is that both the Friend and Rauscher viruses in high titer are initially associated with thrombocytopenia, whereas polycythemia vera is generally associated with thrombocytosis. Both disease states must affect initially the marrow stem cells. Basically we were seeking clues for alterations in megakaryocytopoiesis in preleukemic states as compared to a full-blown leukemic condition such as CML, considered a close relative of PV (*26*). In CML there is a genetic marker—the Philadelphia chromosome—that has been demonstrated not only in the white blood cell precursors but also in erythroblasts and megakarocytes (*27*). Again, this disease is a panmyelosis involving first the stem cell and subsequently the differentiated marrow precursors.

Simultaneous platelet and fibrinogen studies utilizing ^{75}Se have been performed to date in 7 patients with CML, 9 with PV, and 8 with MF. The data, summarized in table 1, reveal that platelet as well as fibrinogen survivals, were significantly longer in CML as compared to PV or MF. Platelet survival was decreased and platelet turnover increased in the PV as compared to both CML and the control data. Mean platelet turnover was increased in both PV and MF and within the control range in CML. Representative platelet survival curves are presented in text-figure 4. The upper panel gives data on PV and MF and the lower an CML. The platelet survival is calculated as the time in days from the 50% point on the ascending slope to the 50% point on the descending. It is apparent that platelet survival is shorter in PV and MF (*28, 29*).

Clinically early CML is rarely associated with severe hemorrhage or thrombosis and this was true in the present series. Serious thrombotic episodes were noted in 7 patients with PV and MF; another had recurrent rectal bleeding. We postulated that the platelets produced by patients

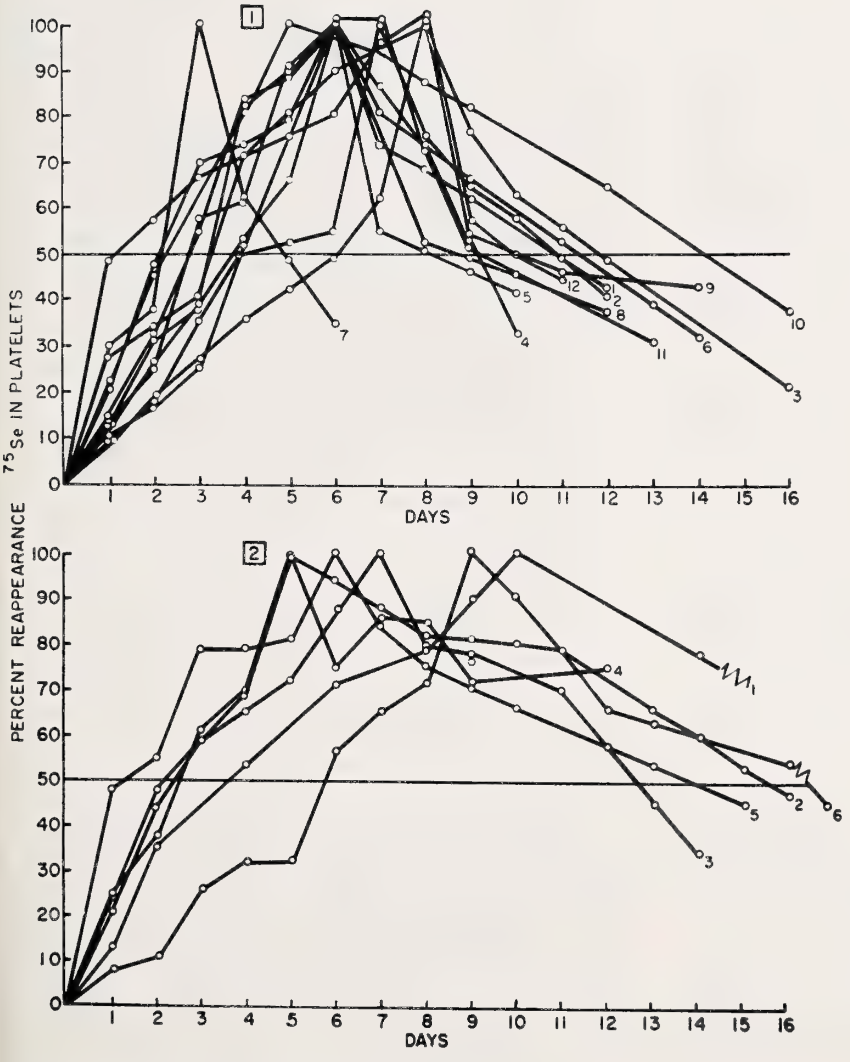
TABLE 1.—*Platelet and fibrinogen kinetics in chronic myeloproliferative disorders*

	Platelets			Fibrinogen		
	Count (platelets/µl)	Survival (days)	Turnover (platelets/µl/day)	Concentration (mg/ml)	Survival (days)	Turnover (mg/ml/day)
CML*	†(7)					
Mean	402, 000	13. 2	31, 700	271	12. 8	0. 24
Range	218, 000–715, 000	10. 5–16. 0	15, 600–51, 100	206–350	6. 5–19. 0	0. 11–0. 39
PV	(9)					
Mean	536, 000	7. 2	75, 700	219	5. 6	0. 44
Range	354, 000–747, 000	5. 5–8. 5	41, 700–122, 000	162–340	3. 5–7. 0	0. 25–0. 97
MF	(8)					
Mean	1, 094, 000	6. 6	176, 800	318	6. 8	0. 52
Range	56, 000–3, 353, 000	4. 0–11. 5	9, 600–609, 600	233–373	4. 0–9. 0	0. 30–0. 85
Controls	(10)					
Mean	378, 000	10. 6	38, 000	318	7. 8	0. 42
Range	168, 000–475, 000	7. 0–11. 0	22, 000–52, 000	225–400	6. 5–9. 5	0. 33–0. 49

*CML=chronic myelogenous leukemia; PV=polycythemia vera; MF=myelofibrosis and myeloid metaplasia.
†Number of patients studied is shown in parentheses.

with CML are functionally abnormal and underutilized as compared to those of control subjects and to patients with PV and MF. In general, platelets in the chronic myeloproliferative disorders (PV, MF, and CML) are metabolically and functionally abnormal (*28–32*). Platelet aggregation is generally decreased. The thrombocytosis of CML results from decreased platelet utilization rather than overproduction. On the other hand, in the "preleukemic states," i.e., PV and MF, platelet production is increased as is platelet turnover. This overutilization of platelets, when associated with an increased turnover of fibrinogen, is probably a major factor in explaining the increased incidence of thrombosis in PV and MF and perhaps the associated hemorrhage (*28*, *29*).

The previous conclusions are in part confirmed by the studies of Lundin et al. (*33*). In PV, morphologic studies of the bone marrow show that the megakaryocytes are increased in number, size, and volume, which indicates increased production of platelets. In CML, the number of megakaryocytes are less than PV but greater than the controls. However, size and volume of the CML megakaryocyte are less than those in the controls. Therefore, the normal to increased platelet counts in CML can only be explained by prolongation in survival due to underutilization rather than overproduction as in PV. It also suggests a more severe disturbance in megakaryocytopoiesis with progression of the neoplastic process. The situation vis-à-vis platelet survival in PV is like that described in the preleukemic phase of Rauscher virus infec-



Text-figure 4.—Platelet survival curves: 1) *Upper panel*—PV and MF. 2) *Lower panel*—CML. Platelet survivals are calculated as the time in days from the 50% point on the ascending slope to the 50% point on the descending slope of the survival curves. Reprinted, with permission, from *Cancer* (*29*).

tion. However, in the preleukemic phase of AKR leukemia, platelet survival is normal (*16*).

The above observations indicate that the megakaryocyte and platelet are important factors in the pathogenesis of both murine and human leukemia. If human leukemia is caused by a type-C virus particle, then the megakaryocyte may be a major reservoir of the virus or viral oncogene. Subtle changes in megakaryocytopoiesis, platelet count, platelet kinetics, and platelet metabolism may be one of the earliest clues that a preleukemic state exists. Eventually, as the leukemic process becomes fully established, severe alterations in megakaryocytopoiesis and thrombocytopenia occur, leading to severe and frequently fatal hemorrhage.

The increasing awareness of the preleukemic state, particularly regarding such "non-neoplastic" cells as the megakaryocyte, raises the obvious question of whether leukemia is indeed a true neoplasm or rather the result of replicative abnormalities due to external regulatory influences such as infection with a "switched-on" C-type virus. Our entire approach to therapy of the human disease is at present based on the neoplastic or two-population (clonal) theory. The major argument in favor of this theory is the long-term remission and potential cures achieved presumably by eradication of the leuekmic clone (*34*). One might also argue that the antileukemic therapy modulated or interfered with viral replication and permitted the immune system to further control abnormal viral transformation. The preleukemic state, especially as related to the megakaryocyte, is more consistent with the one-population or nonclonal theory of leukemia.

The development of better techniques for the early identification of such a preleukemic state will have important implications, not only in regard to etiology but also to more effective therapy and perhaps even prevention of the leukemic process.

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ADDENDUM

Following the publication of the above Editorial, an intensive program was initiated at Hahnemann to isolate a retrovirus from platelets of patients with chronic myeloproliferative disorders, i.e., PV, essential thrombocythemia (ET), and CML. Platelet homogenates were analyzed by the combined assay technique, during which the 1.17–1.19 fraction of the gradient was subjected to critical point drying for electron microscopy (EM) and biological assay for the RNA-directed DNA polymerase (RDDP). On the basis of the particle counts, the estimated total virus present in patients with PV or ET was estimated at greater than 2×10^5 virus/g of platelets. Both exogenous and endogenous RDDP activities were found at a characteristic density for retroviruses, i.e., 1.17–1.19 g/cm^3. No activity was seen at comparable densities in platelets from normal volunteers or patients with benign thrombocytosis. Furthermore, electron micrographs of thin sections of these platelets revealed particles with retroviral morphology. Not all patients with ET or PV showed evidence of a virus. Viral activity coincided with chromosome abnormalities in the bone marrow. Chromosome patterns were characterized by 30–50% aneuploidy. Recently, we reported a more specific chromosome abnormality, a deletion of part of the long arm of the #21 chromosome (21q−). In 30 patients studied to date, the presence of RDDP activity and retrovirus particles in their platelets correlated with chromosome abnormalities in the bone marrow and particularly with the 21q−. The level of correlation between the 21q− and the presence of viral-like activity is virtually 100%. Treatment of patients with PV and ET with busulfan results in a loss in retroviral activity, preceded by a density shift from 1.17 to 1.24 that we interpret as a loss of the virus lipoprotein coat. These events occur while the platelet count is still elevated and thus can be used as an index of an impending hematologic remission. After busulfan treatment, the chromosome abnormalities in the bone marrow are no longer evident. In CML, unlike PV and ET, RDDP activity occurs in platelets but intact viral particles cannot be detected. This is consistent with our hypothesis that myeloproliferative disorders represent a spectrum of neoplastic diseases. A tissue culture line from the spleen of a patient with myelofibrosis and myeloid metaplasia was established in our laboratory. This spleen showed histopathologic evidence for extramedullary hematopoiesis, contained the 21q− chromosome, and became transformed after 9 months in culture. We are now attempting to grow the putative human retrovirus in this transformed cell line. Now in its 20th passage, the culture exhibits retroviral activity. We are attempting to characterize the retrovirus immunologically and by molecular hybridization techniques. These observations support our contention that, in preneoplastic states, the megakaryocyte and platelet may be an important source for the isolation of a human retrovirus. It is well established that PV can be considered in preneoplastic and, in many circles, a neoplastic disorder on the basis of studies on glucose-6-phosphate dehydrogenase that indicate a clonal evolution. Furthermore, recent data indicate that treatment of PV with an alkylating agent, chlorambucil, results in an increased incidence of acute leukemia and solid tumors.

Guest Editorial

John H. Elder,[1] James W. Gautsch,[1]
Fred C. Jensen,[1] *and* Richard A. Lerner[1]

JNCI 61:625–638, 1978

Multigene Family of Endogenous Retroviruses: Recombinant Origin of Diversity[2,3]

SCOPE OF THE PROBLEM: THINKING IN TERMS OF GENE FAMILY

Although some retroviruses are transmitted horizontally (*1*), most are inherited according to mendelian expectations (*2*). Whereas germ line proviruses can be considered simply as intermediates in the life cycle of infectious agents, this is becoming clearly a limited way of conceptualizing the problem. Rather, we should think in terms of an extensive gene family in which only some members have extrachromosomal potential. Thus we can accommodate interactions and expressions of proviral genes that are rarely, if ever, found as complete infectious particles. As for other multigene families, such as immunoglobulins (*3*), there are a number of properties of members which are of great interest and thus form part of the focus of this paper. Here we will consider the *general problems* of: *a*) the extent of the gene family, *b*) the relatedness of family members, and *c*) regulation of expression of member genes. However, the proviral gene family is unique insofar as some members have extrachromosomal potential. This leads us to consider the *special problems* of: *a*) acquisition of new family members by the organism, and *b*) high-frequency recombination between family members.

We can now consider the above-listed properties of this multigene family in detail. To formalize the concept, we comment here that the interplay between an extensive gene family, the members of which may recombine with high frequency, opens the possibility for a generation of an almost infinite diversity of viruses and viral gene products. This theoretic formulation predicting extensive generation of diversity has recently been verified for the viruses associated with the spontaneous AKR lymphoma (*4*).

EXTENSIVE GENE FAMILY IN THE MOUSE

Various ways exist for "counting genes," the most common being via nucleic acid hybridization. Several studies were done, in which a variety of probes were used to count retroviral genes in the mouse (*5–7*). All these studies indicate multiple proviral genes in the genome—but how many? Some idea of the extensiveness of the family can be ascertained by the following considerations. If we subdivide the various endogenous viruses using functional or morphologic criteria, we find several classes of provirus in the mouse genome. For example, a mouse may have, in the genome, proviruses related to three different host range classes of virus (ecotropic, xenotropic, and amphotropic) as well as mammary tumor virus and sequences related to a virus expressed in the Asian mouse (*8*). One assumption is that, for every sequence we score, a complete provirus exists in the genome. This gives five classes of provirus, each containing 10–12 copies of 6.0×10^6 daltons of DNA or as much as 0.03% of the mouse genome. Even if the number is incorrect by severalfold, the point is that the gene family is already extensive, but with untold complexity. For technical reasons, to date we have only counted genes that in their extrachromosomal phase give rise to viruses with sufficiently distinct biologic properties so as to be considered unique. Thus highly divergent proviruses not giving rise to infectious viruses were not counted. The problem is akin to trying to score variable region genes of immunoglobulin by means of a constant region probe.

MEMBERS OF PROVIRAL FAMILY CONSISTING OF CONSTANT AND VARIABLE CISTRONS

Recently, rapid methods have been devised to isolate multiple distinct viruses and determine the primary structural relatedness between the various gene products (*9–10*). Conventionally, the mouse retroviral genome is depicted as in text-figure 1, in which *gag* is the group antigen gene, *pol* is the polymerase gene, *env* is the envelope gene, and C is the constant region with an unknown coding capacity. The products of the *gag* gene are four proteins with molecular sizes of 10,000, 12,000, 15,000, and 30,000 daltons; the *pol* gene encodes the 70,000-dalton reverse transcriptase; and the *env* gene encodes a 70,000-dalton glycoprotein (gp70), of which approximately 45,000 daltons is the primary protein gene product and 25,000 daltons is carbohydrate. Additionally, the *env* gene encodes a hydro-

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ABBREVIATIONS USED: MCF=mink cell focus-forming; MuLV=murine leukemia virus(es).

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[2] Received December 9, 1977.

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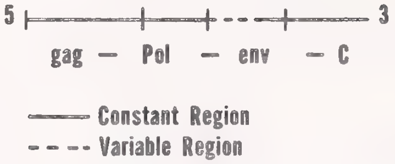
Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.



TEXT-FIGURE 1.—Gene order of RNA tumor viruses. Representations of gene lengths are intended as schematic and are nonproportional. The terms "constant" and "variable" are, as with immunoglobulins, relative. *gag:* Gene encoding internal core porteins p30, p12, p15, and p10; *Pol:* RNA-dependent DNA polymerase gene; *env:* gene encoding the envelope protein gp70 as well as the conserved p15E protein; C: constant portion of unknown coding capacity.

phobic 15,000-dalton protein (p15E) that is probably bonded by disulfide bridges to gp70 (*11*).

Another instructive way of considering the viral genome is in terms of constant and variable portions relative to individual members of the multigene family. Two important points are: 1) Certain cistrons of the viral genome are highly conserved, whereas others are variable; and 2) variable cistrons can, in turn, be divided into constant and variable domains. Let us begin with the variable gene of the 3′ end of the genome that encodes gp70. We can operationally assign a constant region to the gene insofar as a single antiserum can be raised that will react with every gp70 molecule thus far recovered from inbred and wild mice, with the possible exception of the gp70 molecules of Asian mice. In contrast to the immunologically defined constant regions of gp70, analysis of tryptic peptides has shown, with one exception (*see* below), that no two gp70's isolated from different viruses are identical; thus the molecule has variable portions. In figure 1 the tryptic peptides of four distinct gp70 molecules are shown. Each gp70 has distinct tryptic peptides which allow classification and subdivision of the viruses, according to relatedness. In studies of over 50 different virus isolates (*9*) gp70 molecules were grouped according to their structure. Although the molecules were grouped solely according to structure, they fell naturally into subfamilies according to host range groups. This is perhaps not surprising, since the variable portion of the gp70 molecule presumably plays a role in the specific cellular recognition that determines host range. Thus we can now picture the major virus envelope glycoprotein gp70 as a molecule consisting, at some level, of constant and variable portions. Organization of the constant and variable regions of gp70 should be considered. For some time we have speculated that these regions are organized into domains, as for the immunoglobulin molecule (*9*). Thus the variable gp70 domains allow for the specificity in virus-to-cell recognition. Recently, Vogt and his colleagues (personal communication) have completed studies that strongly support the concept that the *env* gene cistron of avian viruses is divided into constant and variable domains.

In contrast to gp70, p30, which by mass is the major core protein, is highly conserved. The issue may be stated simply that two viruses with highly divergent gp70 molecules may have virtually identical p30 molecules. This is illustrated in figure 2 in which the gp70's and p30's of six different viruses are compared. Yet not all p30's are identical, and thus far we have recognized four types in the mouse (*see* below; also fig. 3). Although p30 has four types, the picture that emerges is a highly variable *env* gene linked to a *gag* gene, at least part of which is highly conserved.

We know less about the relative structure of the polymerases p12, p10, and p15, but evidence suggests that polymerases p10 and p15 are relatively conserved, whereas p12, in accord with its specific RNA-binding function, is more variable (*12, 13*). After consideration of all the data, a concept emerges of the retroviral genome according to the scheme in text-figure 1: The genome can be divided into constant and variable regions, and a single cistron like that of *env* may have constant and variable domains. Inasmuch as we refer to endogenous viruses, when we define a system of constant and variable cistrons for the virus we have, by definition, uncovered a like system in the genome.

EXPRESSION OF MULTIPLE DIVERGENT PROVIRUSES IN A SINGLE MOUSE

A count of the genomic equivalents of a multigene family is one matter, but their expression is quite another. In a family like that of the immunoglobulins, an animal may express around 10 mg/ml of antibody protein representing 10^6 distinct gene products. (In the antibody system, the unresolved question is whether all family members are present in the germ line or whether they are generated by somatic recombination.) In the retrovirus system, two very important aspects of proviral expression, which only recently have been recognized, are: *a*) Proviral expression is regulated during differentiation and development, as for other host genes, and *b*) multiple divergent proviruses are expressed in a single mouse.

Using immunofluorescence microscopy with a monospecific antisera to gp70, Lerner et al. (*14*) demonstrated a remarkable restriction of proviral expression. As can be seen in figures 4–6, the presence of gp70 is largely confined to lymphoid and epithelial cells, in which gp70 may be present in large amounts. By studying congenic pairs of mice in which one member of the pair is known to lack gp70 expression, one can prove that the fluorescence is the result of proviral expression. An immunofluorescence study of the epididymis of one such pair—129 and the congenic 129 G_{IX}^- mice—is shown in figure 6. The 129 G_{IX}^-, which differs from the 129 G_{IX}^+ by a single gene, lacks gp70 and immunofluorescence reveals that proviral expression is completely absent from the epididymal epithelium. [Through parallel studies of gp70 and viral related alloantigens such as G_{IX}, the regulation of proviral expression during differentiation was discovered. The historical and experimental factors cannot be adequately described here but can be ascertained from (*14, 15*).] Thus we see that proviral gene expression is regulated during differentiation. However, this does not mean that the provirus plays a role in differentiation.

Inasmuch as we are concerned with a gene family in which some members are expressed, the next question is: How many distinct proviruses are expressed in a single mouse? Again, until we isolate gp70 from each site of differentiation, we cannot know the exact number; however, we already know that multiple distinct gp70's are expressed in a single mouse. As a paradigm, let us consider the AKR mouse. In the AKR we know of 5 members of the gene family: two ecotropic virus genes [*Akv-1* and *Akv-2* (*2*)], one AKR xenotropic virus gene, one gene for serum gp70, and one gene for the gp70 secreted into the male genital tract (*9*, *16*). The striking fact that they are all different establishes that multiple proviruses are expressed in a single mouse. Although *Akv-1* and *Akv-2* are located on different chromosomes, their gp70's appear identical, perhaps suggesting that duplication of this gene is a recent event. The nature of the serum gp70 deserves special emphasis. A number of mice have high levels (20-50 μg/ml) of gp70 in the sera. Free gp70's isolated from sera of different mice are shown in figure 7. Although clearly related in structure, all gp70's are distinct, presumably reflecting a polymorphic allele or different gene products. When the serum gp70's were first discovered, there were two ways of conceptualizing their relationship to "actual viral" gene products. Because the proteins were originally detected immunologically, one view was that they were simply host proteins cross-reactive with viral gene products. However, this concept became less tenable when it was discovered that at least one virus [one of the NZB xenotropic viruses (*9*)] has as its major envelope gp70, the serum protein (fig. 8). Thus the problem is best considered in terms of a set of proviral genes that are usually only expressed as serum proteins but that, under some circumstances, can serve as viral envelope constituents. Therefore, one should consider how to define viral proteins versus host proteins that can be used functionally by the virus and yet appear as regular structural components of complete particles. This arguable distinction leads to a philosophical issue. In terms of multigene families, our viewpoint is that the proteins we describe are encoded by germ line proviruses, all of which may, under some circumstances, encode complete particles. Therefore, this view places much emphasis on host regulation of proviral expression.

HOST CONTROL OF PROVIRAL EXPRESSION

As stated above, proviral expression is strictly regulated by the host cell. This is perhaps not surprising because the proviruses are, in fact, host genes. A different approach to the problem is in relation to the extensive gene family. Many distinct mouse retroviruses exist, each of which presumably has a proviral complement in the germ line. Thus we are concerned with either enormous polymorphism or a constant genotype expressing variability via regulatory genes. Although the issue is not settled, recent evidence suggests that most ecotropic, xenotropic, and amphotropic proviruses are present in the genomes of all wild mice and inbred mouse strains and that regulatory genes control which one(s) will be expressed (Rowe WP: Personal communication).

ERECTOR SET OF VIRAL GENES

We now know of the existence of a large gene pool in the murine germ line, only some members of which are expressed as either differentiation proteins or complete viruses. Thus we now can consider the reassortment of genes and can document two basic processes, which may be termed intracistronic and intercistronic recombinations. The intracistronic recombination has enormous consequences for a generation of virion diversity (discussed separately below).

Here let us take up intercistronic recombination. Given a family of genes encoding such factors as envelope proteins and internal components, one can think of construction of an infectious particle as a kind of erector set exercise in which one or another envelope is associated with one or another internal component to make a particle with a certain host range (and, e.g., *Fv-1* restriction). With the elucidation of good structural markers for the various virion components, intercistronic recombination has recently been shown probably to be a frequent occurrence in that it is possible to demonstrate a given set of internal proteins associated with two different envelopes to construct biologically distinct virions. For example, as mentioned above, we have markers for four different p30's of the mouse (fig. 3). Figure 2 shows three types of endogenous p30 associated with two different envelopes; thus six biologically distinct particles may be constructed. Therefore, an assortment of viral genes clearly exists that offers a genetic flexibility heretofore not seen in eukaryotic systems. We have begged the issue as to when the recombination has taken place. The two extremes would be that *a*) the various modular components exist as free entities in the genome and are assembled during replication and *b*) the various proviral genes have previously undergone recombinational events and those we now observe are stable, distinct, integrated products. If we envisage reassortment of genes and integration of the new construct, then we must assume that, if integration occurs in germ cells, the murine genome will increase in size due to addition of the new proviruses. This modular or erector set concept is reminiscent of the protovirus concept of Temin (*17*).

GENERATION OF VIRION DIVERSITY BY VARIABLE GENE RECOMBINATION

One of the most remarkable outgrowths of the studies of the proviral gene family has been the recent finding that, during the neoplastic process, recombination between the variable or *env* genes of endogenous proviruses occurs, leading to generation of a virion diversity so that even in inbred strains the tumors of individual mice each have genotypically distinct viruses. Because the proviruses encode surface glycoproteins of the thymocyte, the further implication of the *env* gene recombination is that the surface of each tumor has at least one set of unique cell surface molecules. Thus, conceptually, the main interest in these newly recognized viruses is in the generation of virion diversity. The viruses of the spontaneous lymphomas of AKR have been termed MCF virus (*18*) to call attention to their distinctive cytopathic effect on mink cells.

Other features of the MCF viruses exist that are germaine to the present discussion. First, the presence of MCF viruses appears to be particular to the leukemic or preleukemic state in that they cannot be isolated from normal mice. Second, the host range of the MCF virus has been extended in that they infect both mouse cells (ecotropic) and cells of other species (xenotropic) (*18*). These viruses are called dual tropic rather than amphotropic to distinguish them from the broad host range viruses of wild mice (*19, 20*). Third, the viruses are leukemogenic: When they are inoculated into AKR mice, the latency period for leukemia is decreased. Also, tumors have been induced by the MCF virus in C3H/Bi mice, and further tests are in progress (Hartley J: Personal communication).

Of all the properties of the MCF viruses listed above, that of dual tropism suggested that they were recombinants in the *env* gene (*18*). [In avian systems, the recombinants isolated to date have the host range of only one of the viruses (*21, 22*) and thus presumably differ from murine recombinants in which two host range markers may be inherited (*4*).] Because we had previously established a library of envelope structures, it was easy to compare the MCF viruses with other known murine agents, particularly those of the AKR mouse (*4*). Figure 9 shows the tryptic peptides of possible parental *env* genes, and figure 10 shows the tryptic peptides of four MCF virus isolates. Two points are clear: Each MCF virus is distinct (thus providing structural evidence for the diversity described above), and the MCF viruses appear distinct from any of the known possible parental viruses of normal AKR mice. The viruses have peptides common to both xenotropic and ecotropic proteins and thus appear to be recombinants (fig. 9). From the tryptic peptide pattern we can establish with certainty that one parental gene is *Akv-1* or *Akv-2*, but the origin of the xenotropic information is uncertain. Inasmuch as the appearance of new peptides was accompanied by the loss of others, trivial explanations for these results, such as presence of more than one virus, appear unlikely.

The next question about MCF viruses concerns the origin of the 5′ end of the genome. To establish this origin, we determined the structure of the *gag* gene products, p30 and p15 of parental and recombinant viruses. A typical result is shown in figure 11. From these data, as well as from other maps not shown, it is possible to establish the *Akv* gene as the donor of the 5′ end of MCF viruses. The picture which then emerges from the origin of the MCF viruses is as follows. *Akv-1* or *Akv-2* recombine with one or more xenotropic viruses to give a new virus in which the entire 5′ end, up to and including part of the *env* gene, is of *Akv* origin. The variability is due to substitution of a portion of a xenotropic *env* gene at the 3′ end of the genome. In different isolates of MCF viruses, varying amounts of xenotropic *env* information are present, thus leading to their individuality.

STATUS OF THE PROBLEM

All the virologic evidence we have summarized provides the molecular basis for complexity of the murine viruses, as well as for the diversity of the cell surface of AKR lymphomas. An important question concerns the extent of the diversity. We recognize that diversity in the viral systems is generated by intracistronic recombination in the highly variable *env* gene of the proviruses as well as reassortment of modular components by recombination between the variable *env* genes with the more constant *gag* genes. The extent of the diversity depends on how many parental genes are involved. If the *env* genes are derived from many members of the large multigene family, the process of recombination probably allows construction of a nearly infinite array of virions.

Finally, some philosophic observations: Inasmuch as the diversity of endogenous viruses has grown, there is a tendency to believe that the problem is too complex or messy for resolution. However, diversity does not complicate the problem—it is the problem. To assume that the problem is too complicated would be like ignoring the antibody problem because of too many specificities.

RECOMBINANT gp70 *LEUK* GENE?

The recent findings that highly oncogenic viruses have recombinant *env* genes without any other apparent alteration in the genome has caused us to raise the issue of whether a recombinant gp70 is directly responsible for leukemogenesis (the *leuk* gene product) (*4*). To put this issue into perspective, one must recall that gp70 is normally expressed on the surfaces of certain differentiated cells, notably thymocytes, which are the oncogenic targets of the natural leukemia of the mouse. To invoke a role for gp70 in oncogenesis, one need not presume that the normally expressed gp70 serves an important function; it is simply necessary to say that, in its unaltered form, gp70 does no harm. As a result of recombination, however, the 5′ end of the *env* gene or the N-terminus of the gp70 molecule is replaced by an inappropriate structure. One can easily imagine how replacement of the N-terminus of an alloantigen might lead to altered growth control and oncogenesis. Within this scenario, one can easily rationalize the strict differentiation dependency of the oncogenic event in that only cells which normally carry gp70 in their membranes or which can be perturbed by an altered gp70 molecule will be transformed by virus. In its broadest context, this concept of the oncogenic event suggests that, as long as structural virion components and/or viral gene products necessary for viral replication remain confined to the replicative pathway of the virus, oncogenesis will not ensue. Yet transformation can occur if they become inappropriately modified and then inserted into the plasma membrane. An example of this process, other than recombinant gp70, may be the glycosylation and insertion of the *gag* polyprotein into the plasma membrane of certain murine lymphomas (*23*, *24*). The difference between these notions and more conventional concepts of virus-cell interactions is that they place the oncogenic event onto inappropriate modification of virion proteins by the cell rather than the reverse.

NOTE ADDED IN PROOF

During the course of this writing, an in-depth study of the structural diversity of the retrovirus core protein p30 has been completed by Gautsch et al. and is now in press (*25*).

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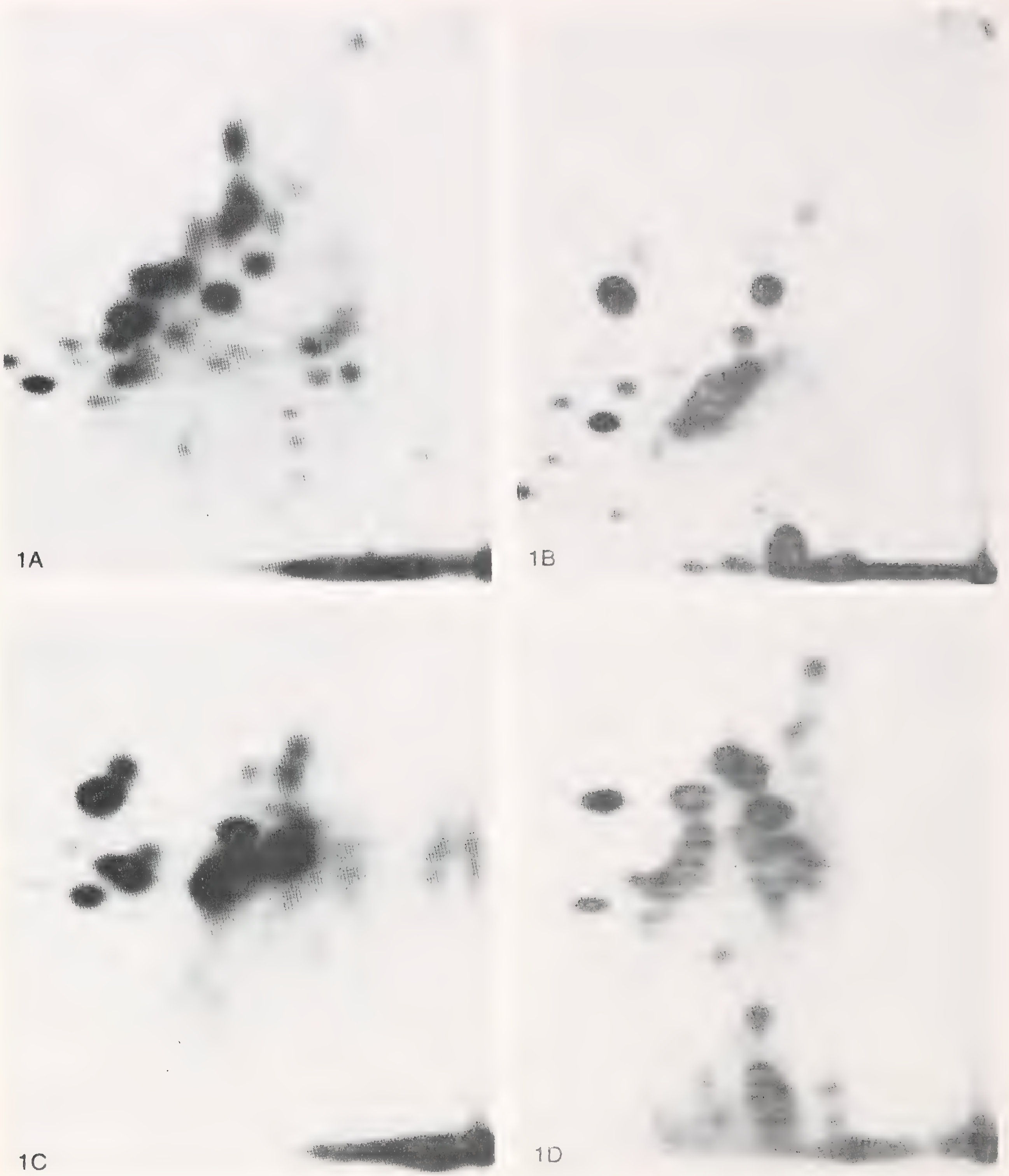


FIGURE 1.—Comparison of the ^{125}I-labeled tryptic peptides of the gp70's of four groups of endogenous C-type viruses. Details of gp70 isolation and tryptic peptide analyses are described in (*9*). A) AKR ecotropic virus gp70; B) C57BL/6 xenotropic virus gp70; C) #11235 wild mouse amphotropic virus gp70; D) 129 G_{IX}^{+} serum gp70, similar in structure to NZB xenotropic virus gp70 (*9*).

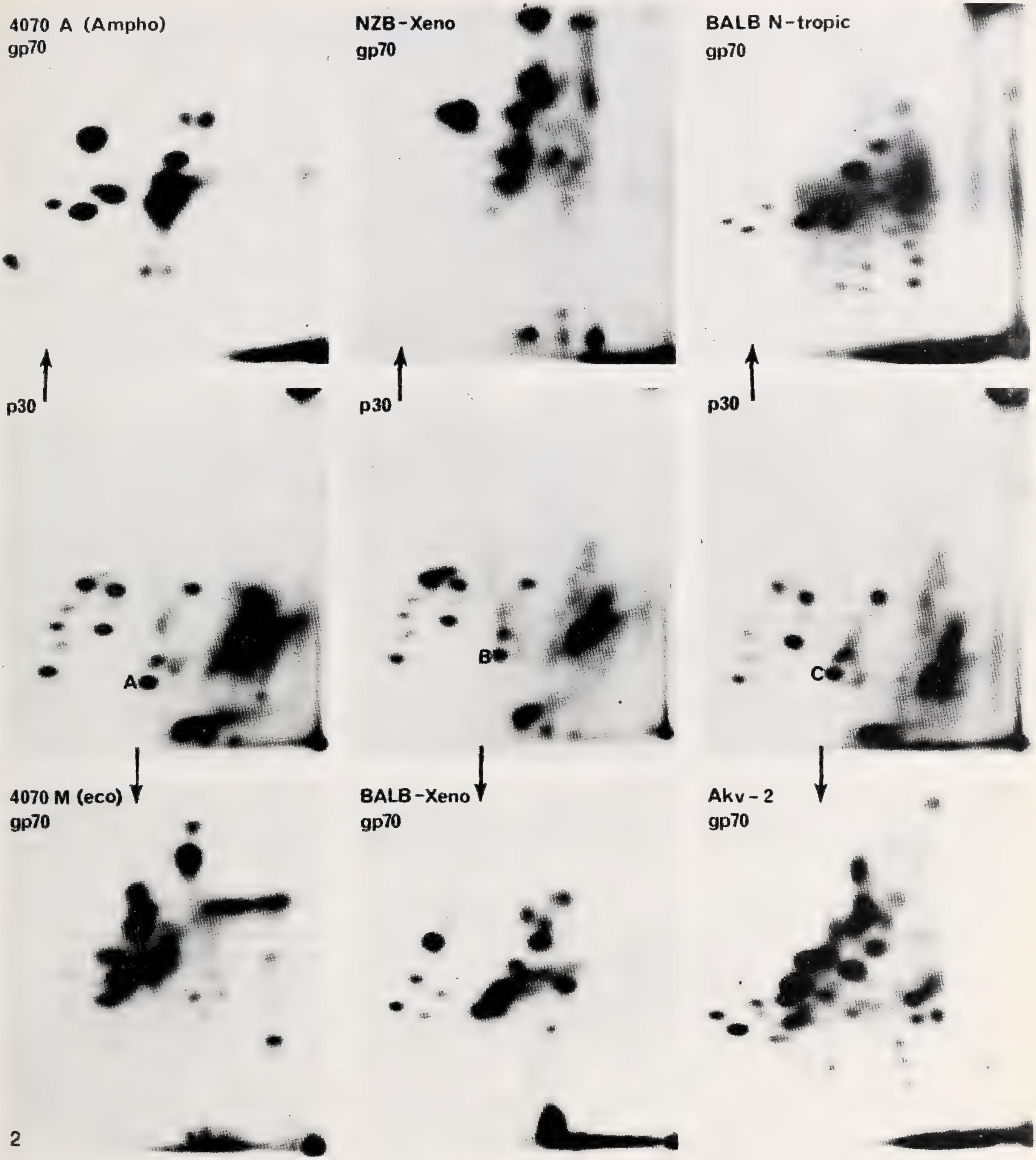

4070 A (Ampho)
gp70
NZB-Xeno
gp70
BALB N-tropic
gp70
p30
p30
p30
A
B
C
4070 M (eco)
gp70
BALB-Xeno
gp70
Akv-2
gp70
2

FIGURE 2.—Maps of p30's of the A, B, and C structural subtypes (*arrows*) with fingerprints of different virus gp70's, illustrating that MuLV that have different gp70's can have indistinguishable p30's. Ampho=amphotropic; Xeno=xenotropic; eco=ecotropic.

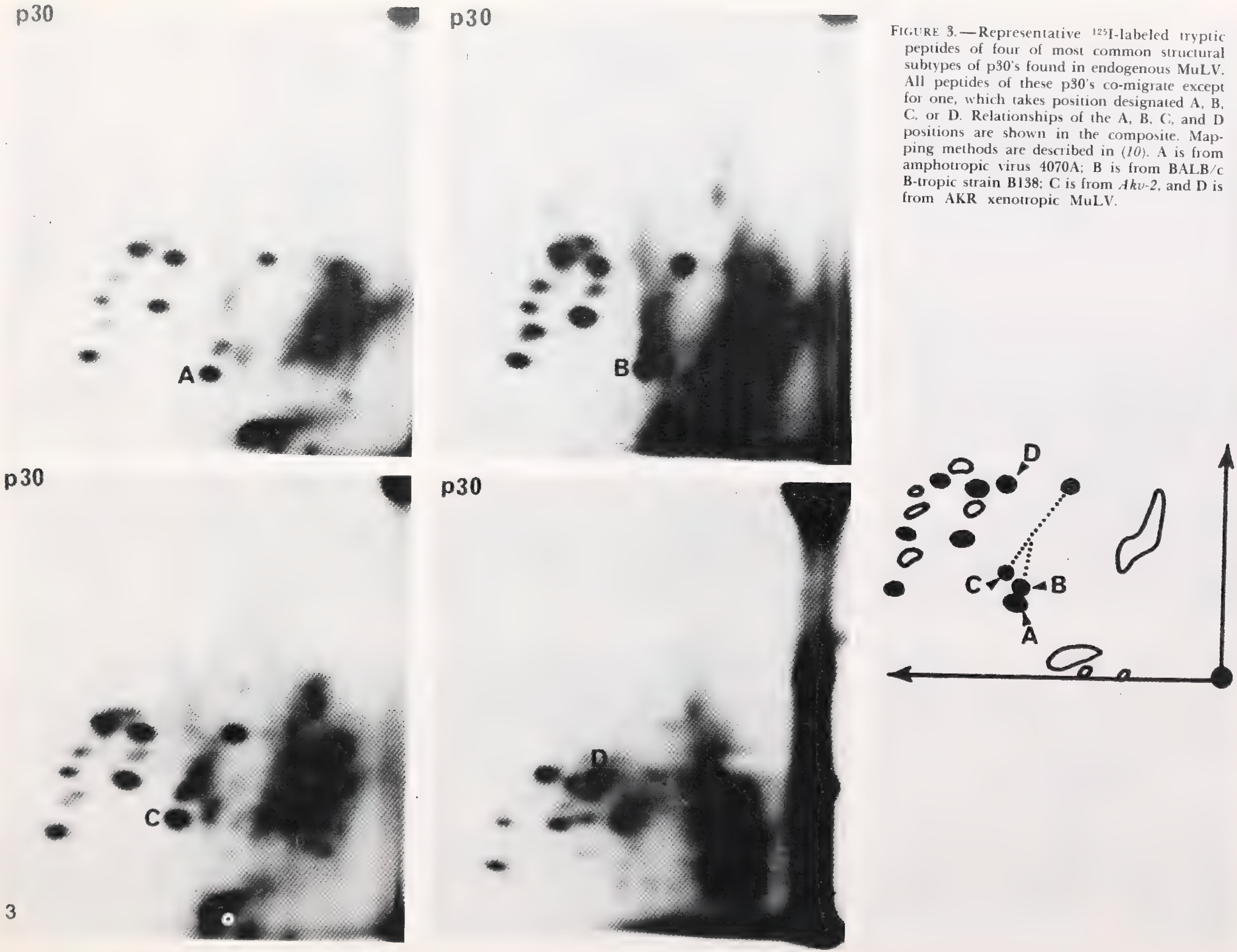
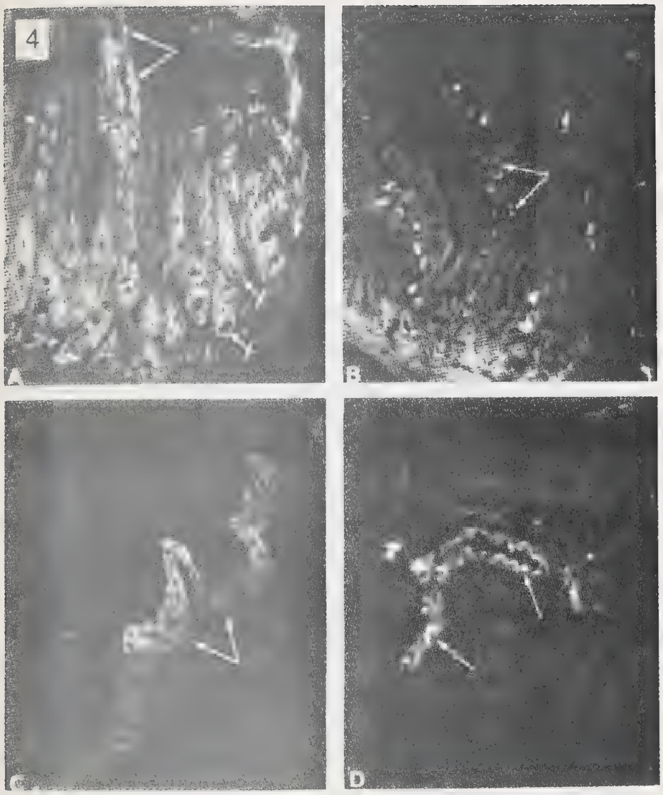


FIGURE 3.—Representative ^{125}I-labeled tryptic peptides of four of most common structural subtypes of p30's found in endogenous MuLV. All peptides of these p30's co-migrate except for one, which takes position designated A, B, C, or D. Relationships of the A, B, C, and D positions are shown in the composite. Mapping methods are described in (*10*). A is from amphotropic virus 4070A; B is from BALB/c B-tropic strain B138; C is from *Akv-2*, and D is from AKR xenotropic MuLV.



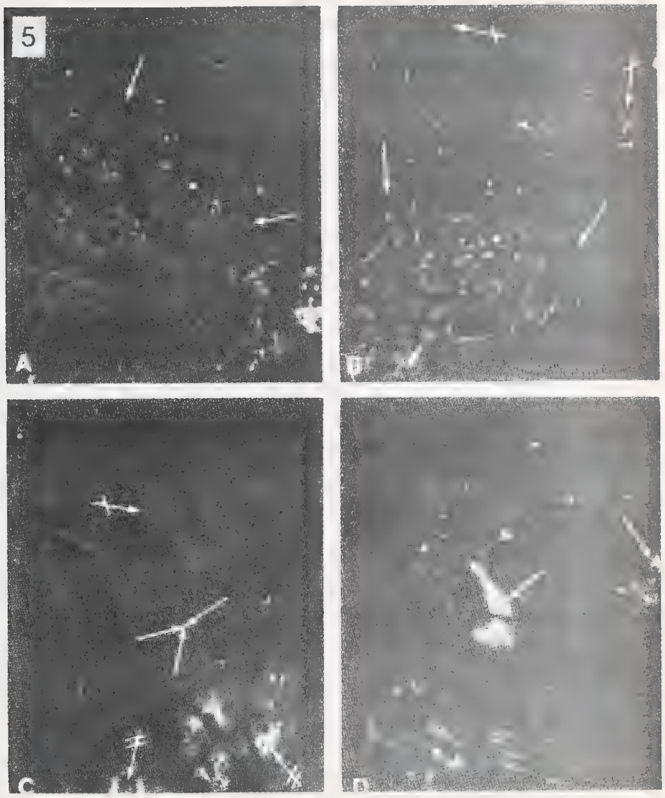


FIGURE 4.—A) Jejunum of NZW mouse with cytoplasmic gp70 staining in the crypts of Lieberkühn (*cross-hatched arrows*). Lamina propria of the villi contained many cells with diffuse cytoplasmic staining (*joined arrows*). B) Jejunum of 129 G_{IX}^+ mouse contained very faint cytoplasmic staining in the crypt area, with only a few scattered cells positive for gp70 in the lamina propria (*joined arrows*). C) Gallbladder epithelium of NZW mouse stained brightly for gp70 (*joined arrows*). D) Kidney of NZW mouse had bright cytoplasmic gp70 staining in scattered tubules with small diameters (*arrows*). In some areas, staining was most intense on the luminal aspect of the tubular epithelium. Indirect immunofluorescence staining with the use of goat anti-Rauscher gp70 antisera and fluorescein isothiocyanate-conjugated rabbit anti-goat IgG. [Reprinted with the permission of the Journal of Experimental Medicine (143:151–166, 1976). Original magnification: A and B, × 160; C and D, × 250]

FIGURE 5.—A) Liver of a 14-day-old C57BL/St fetus had diffuse cytoplasmic gp70 staining (*arrows*). B) Lung, diaphragm, and liver of an 18-day-old NZB fetus. The alveolar lumina stained for gp70 (*hatched arrows*) as did the cytoplasm of the hepatic cells (*nonhatched arrows*). The diaphragm was free of staining. C) Intestine and pancreas of an 18-day-old NZW fetus. Faint cytoplasmic staining for gp70 is seen in the developing crypts of Lieberkühn (*single-hatched arrows*). The muscular wall of the intestine (*opposed arrows*) is free of staining. The luminal aspect of the pancreatic acini is brightly stained (*double-hatched arrows*). D) Thymus of an 18-day-old NZW mouse contained clusters of brightly staining cells in the medullary areas (*arrows*). Indirect immunofluorescence staining with the use of goat anti-Rauscher gp70 antisera and fluorescein isothiocyanate-conjugated rabbit anti-goat IgG. [Reprinted with the permission of the Journal of Experimental Medicine (143:151–166, 1976). Original magnification, × 160]

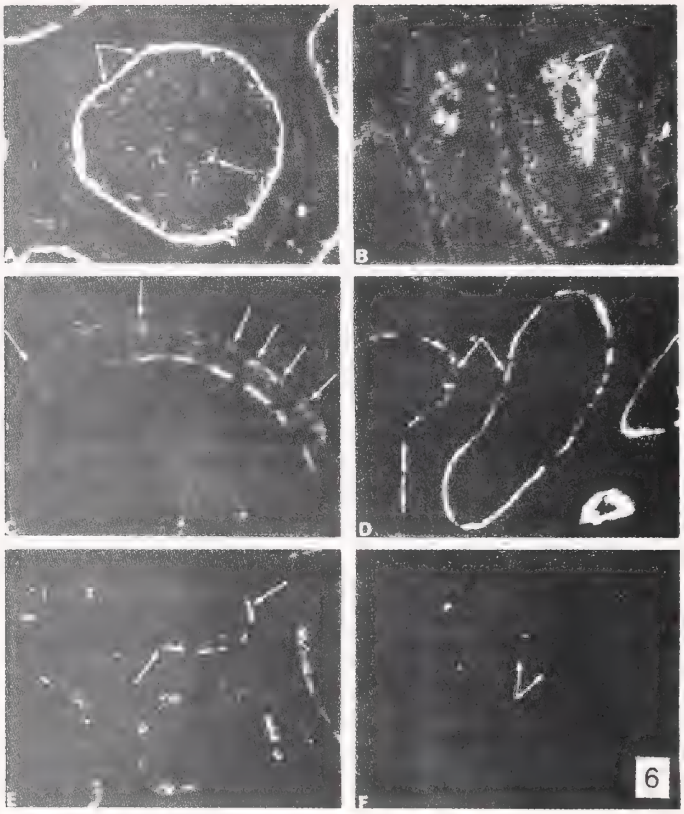


FIGURE 6.—A) Sections of the ductus epididymis from an NZW mouse; *note* accumulations of gp70 along the ciliated luminal (stereocilia) border of the epididymal epithelium (*joined arrows*). The gp70 was also present among the maturing sperm within the lumen of the epididymis (*arrow*). B) Sections from an area of the epididymis near the testis of a mouse; gp70 concentrated in the luminal aspect of the epithelial cells (*joined arrows*). Staining was also present in the cytoplasm of the epididymal epithelium. C) Epididymis of an AKR mouse; *note* scattered epithelial cells with faint gp70 staining (*arrows*) and striking accumulations of gp70 at the luminal aspect. D) Epididymis from a 129 G_{IX}^{+} mouse; gp70 is in the area of the stereocilia (*joined arrows*); but, in contrast to the NZB mouse, accumulation of gp70 was of less magnitude and not continuous around the circumference of the lumen. E) C57BL/St epididymis; small amounts of gp70 are found in the area of the stereocilia (*arrows*). The distribution was strikingly segmental. F) Epididymis of the 129 G_{IX} mouse did not contain detectable gp70. The stereocilia region of the epithelial cell appeared as an empty area (*joined arrows*). Indirect immunofluorescence with goat anti-Rauscher gp70 antisera and fluorescein isothiocyanate-conjugated rabbit anti-goat IgG. [Reprinted with the permission of the Journal of Experimental Medicine (143:151–166, 1976). Original magnification, × 160]

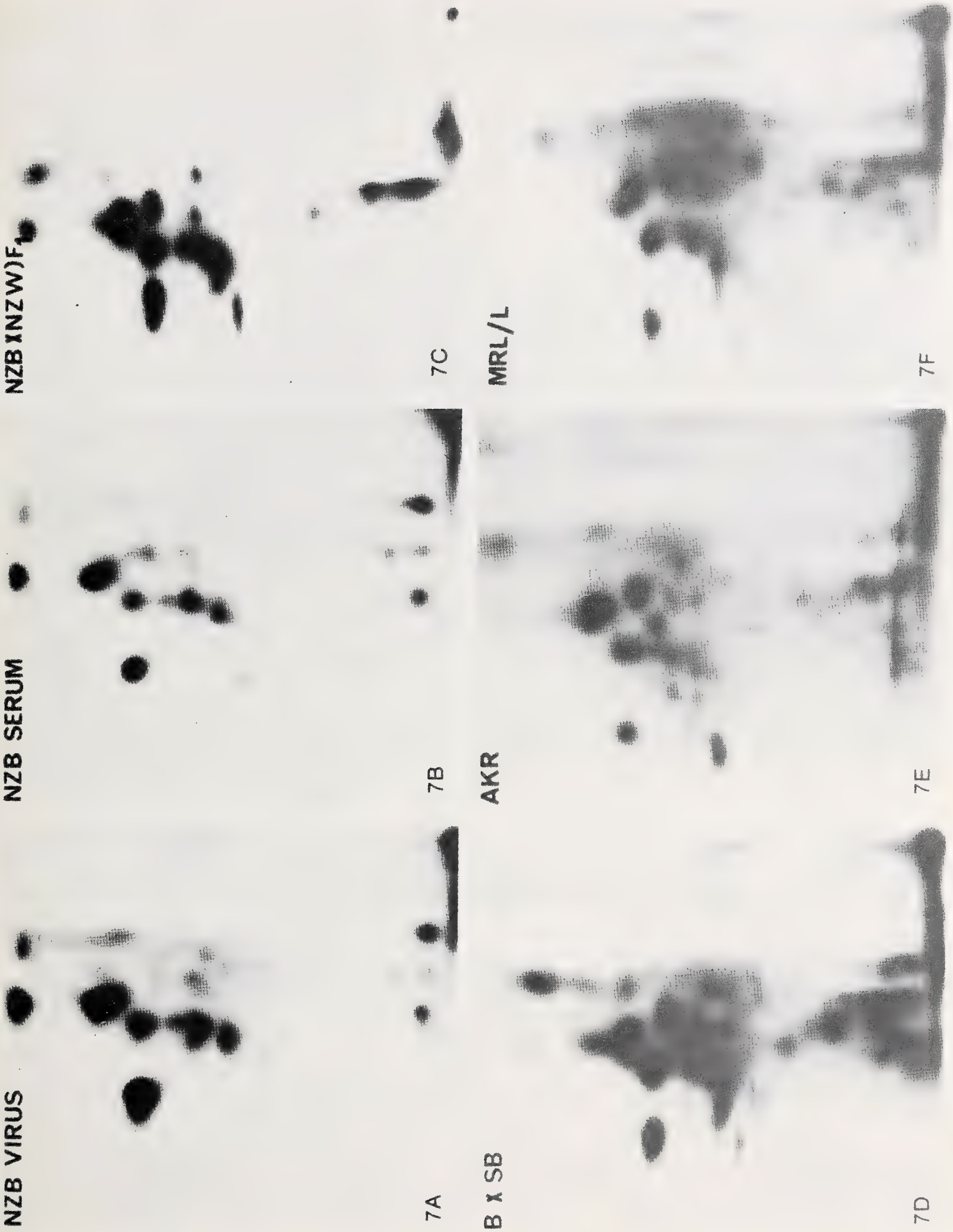

NZB VIRUS
NZB SERUM
NZB x (NZW)F1
7A
7B
7C
B x SB
AKR
MRL/L
7D
7E
7F

FIGURE 7.—Tryptic fingerprint analyses of the gp70's isolated from the sera of several strains of mice. Descriptions of mice as well as isolation and peptide mapping procedure are described in (9).

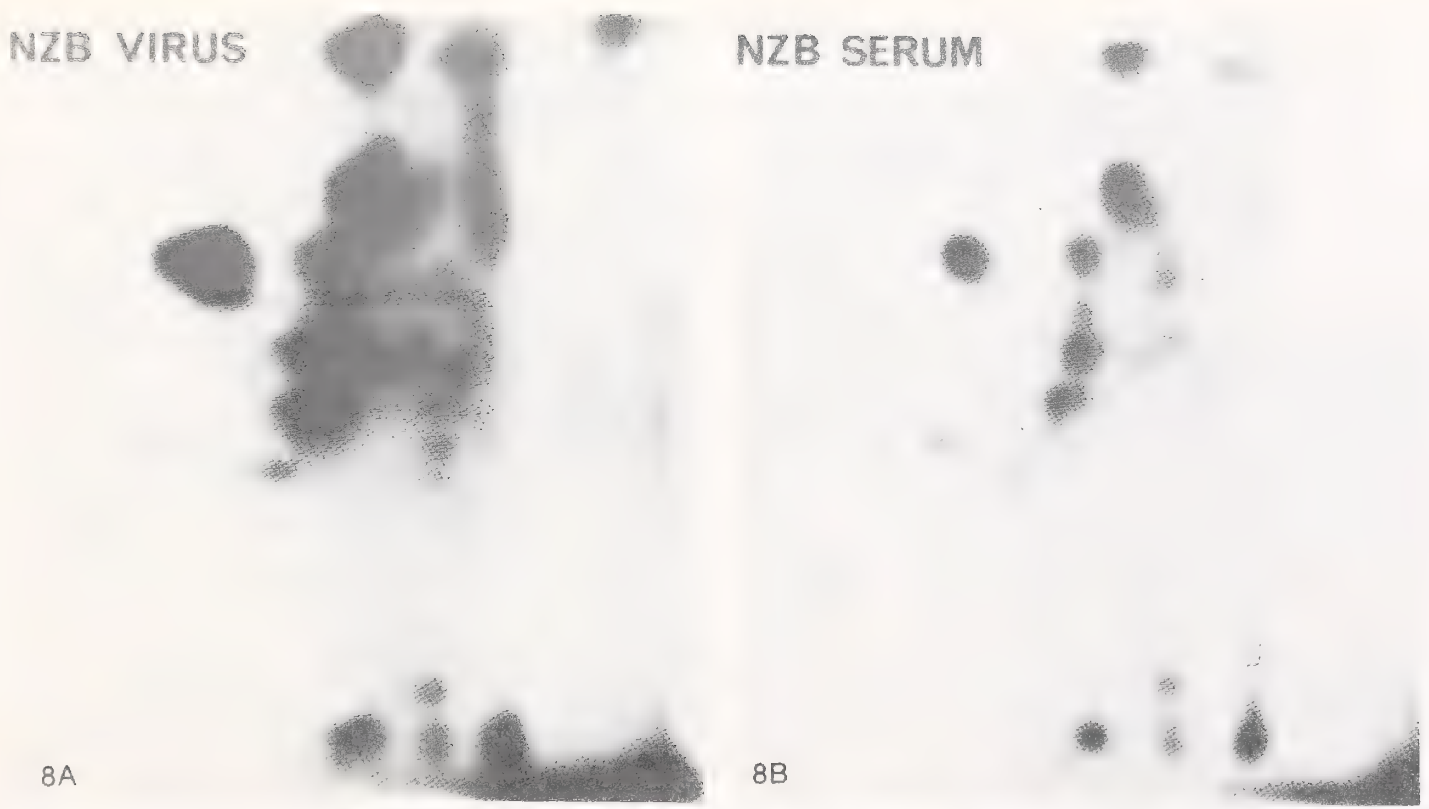

NZB VIRUS
NZB SERUM
8A
8B

Figure 8.—Comparison of the gp70 of NZB xenotropic virus (A) and gp70 isolated from sera of NZB mice (B). Details of gp70 isolation and fingerprint analysis are described in (9).

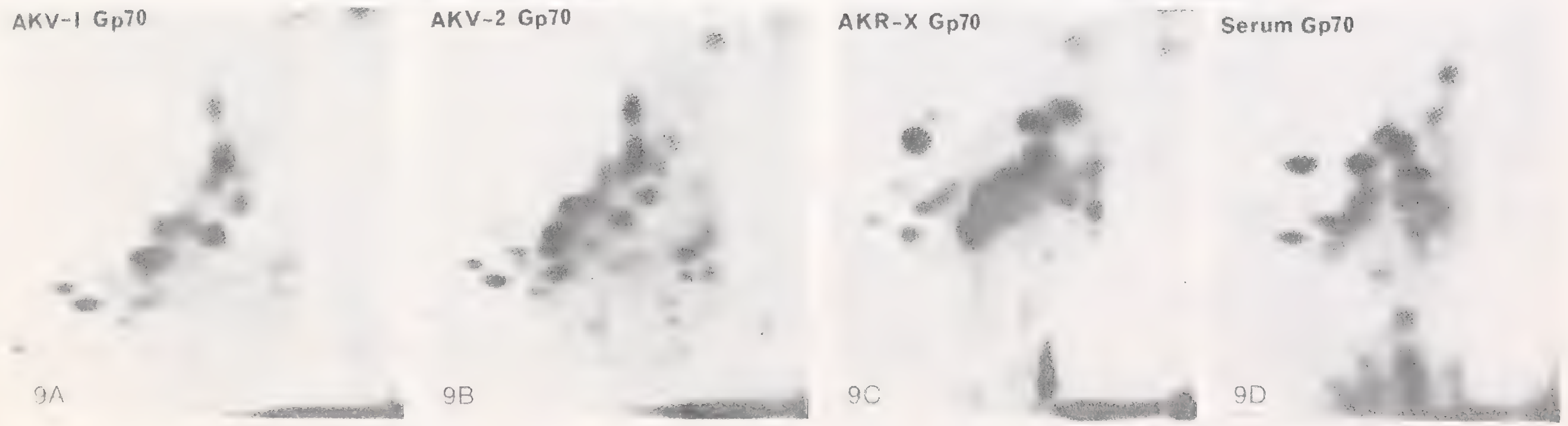

AKV-I Gp70
AKV-2 Gp70
AKR-X Gp70
Serum Gp70
9A
9B
9C
9D

Figure 9.—Tryptic fingerprint analyses of gp70's from known endogenous viruses of AKR. Details of virus isolation, purification, and tryptic peptide analyses are described in (4). The products of the *Akv-1* (A) and *Akv-2* (B) loci are identical (4).

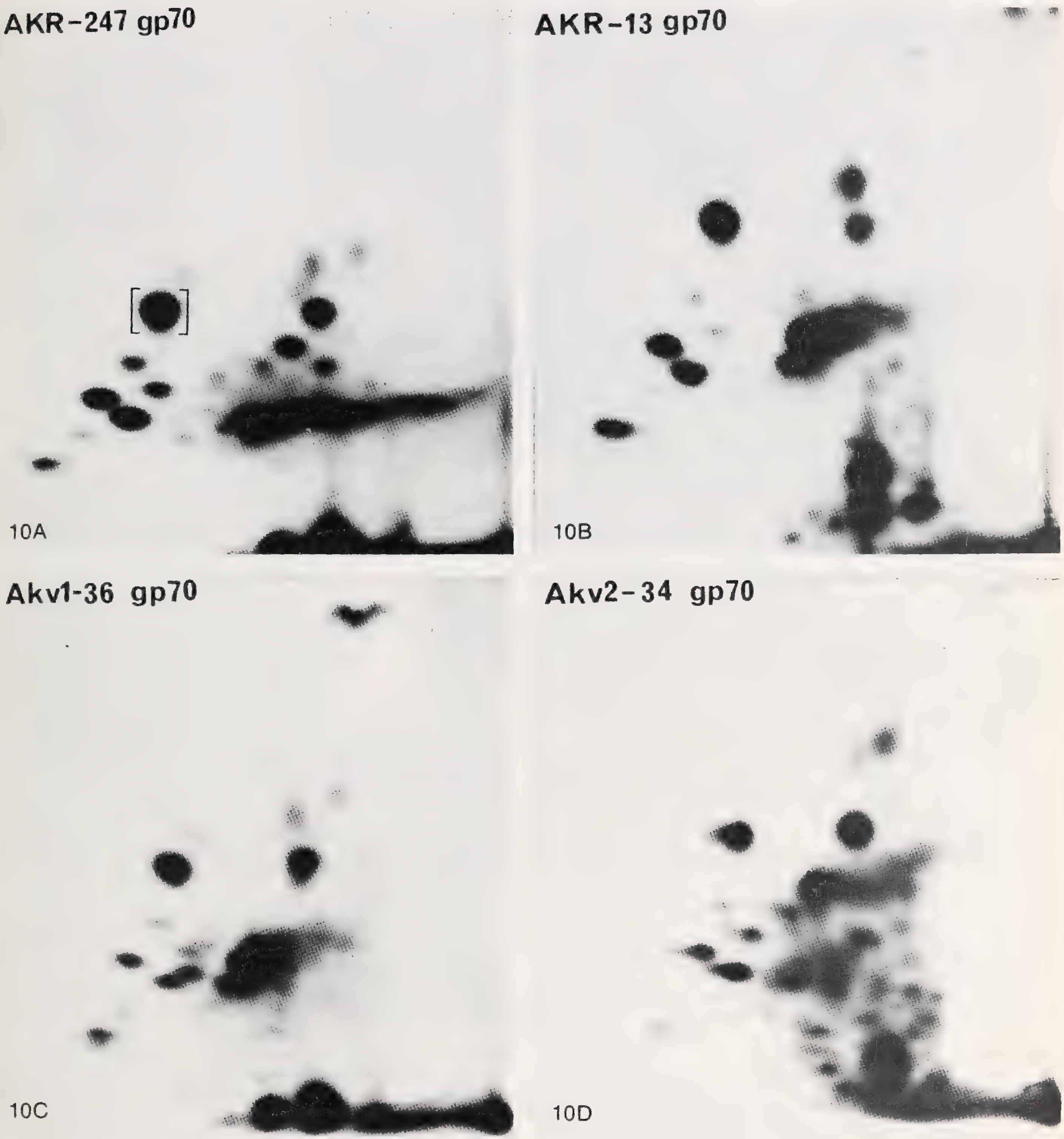
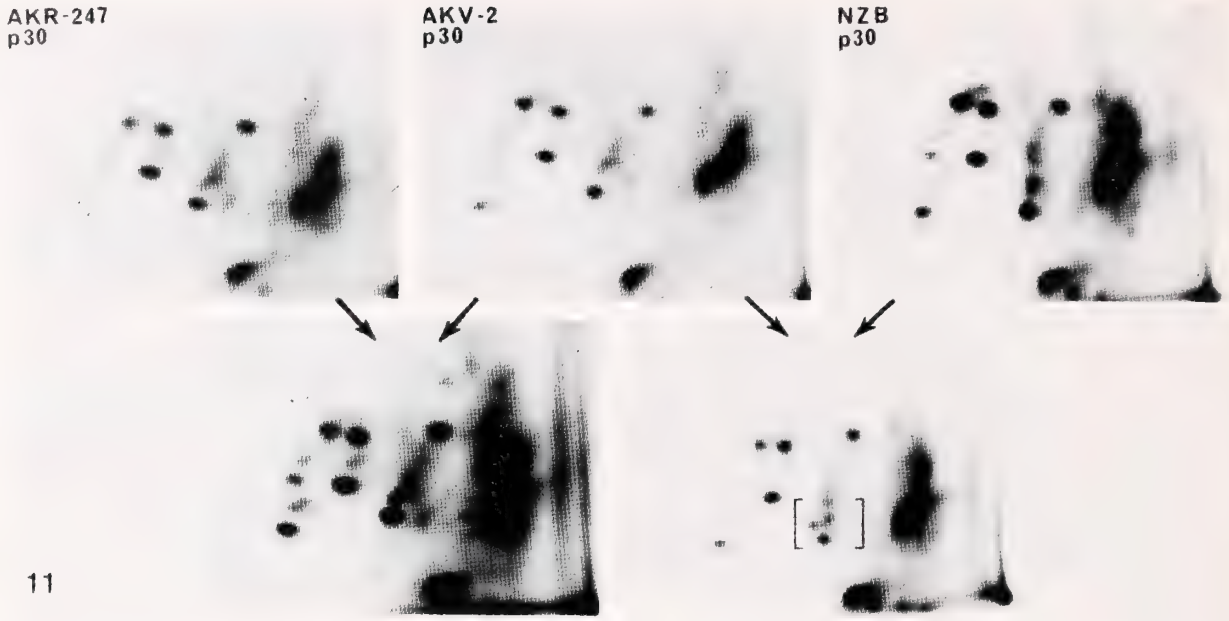

AKR-247 gp70
AKR-13 gp70
10A
10B
Akv1-36 gp70
Akv2-34 gp70
10C
10D

FIGURE 10.—Tryptic fingerprint analyses of four MCF virus gp70's of AKR. Details of virus origins, purification procedures, and techniques of gp70 isolation and analyses are described in (*4, 9*). *Brackets* in 10A denote peptide, present in all the MCF virus isolates; this characteristic is common to xenotropic, but not ecotropic, virus gp70's.



11

FIGURE 11.—^{125}I-labeled tryptic peptides of p30 from AKR ecotropic virus (*Akv-2*), MCF-247, and a xenotropic virus before and after mixing of the peptides to determine relatedness. Mixing of the peptides from *Akv-2* and MCF-247 p30's yielded the same peptide map as that obtained with the separate proteins. A mixture of *Akv-2* p30 and xenotropic virus p30, however, demonstrated differences between the two p30's (*brackets*). NZB virus was used as a prototype xenotropic virus for presentation here.

Guest Editorial — **Pietro M. Gullino**[1]

JNCI 61:639–643, 1978

Angiogenesis and Oncogenesis[2]

The role of angiogenesis in neoplastic diseases has been fully appreciated only since it was shown that a block or lack of angiogenesis could arrest tumor growth. The formation of a tumor as a tissue that grows continuously until the host is killed requires not only the presence of a neoplastic cell population but also a vascular network able to support cell growth. Neoplastic cells acquire the ability to stimulate angiogenesis and thereby sustain their continuous multiplication. As an expansion of this observation, the hypothesis has been developed that capacity to induce angiogenesis was a property acquired by differentiated cell populations on their way to neoplastic transformation. One practical aspect of this hypothesis is that the acquisition of an angiogenic capacity by a tissue that normally is deprived of it could reveal the existence of a "preneoplastic" state and justify prophylactic measures. The theme of this discussion concerns the development of these concepts.

VASCULARITY IN THE EMBRYO

Angiogenesis in most vertebrates arises in the yolk sac as clusters of cells that gradually canalize into a plexus and penetrate the embryo. In the past this initial cluster was considered a specific stem tissue (angioblast) uniquely endowed with the capacity to form vessels (*1, 2*). The local origin of blood vessels as opposed to their derivation from a stem tissue was suggested by the observation that isolated fragments of an embryo became vascularized when separated from the organism before any vessel had penetrated from the periphery (*3*).

After the initial capillary plexus is formed, the vascular system is extended by sprouting, growth, and migration of endothelium (*2, 4*). This appears to be a general rule that is also valid for regenerating tissues (*5*). The development of major vascular pathways from the original plexus occurs predominantly through a process of enlargement and differentiation of capillarylike channels into arteries and veins. With the exception of the largest vessels, the final pattern of arteries and veins emerges from the early network of capillaries through a process of remodeling. Factors that exert a guiding influence on this process are: heredity; growth patterns of parenchymal cells; concentration of metabolites such as O_2 and CO_2; and physical forces such as the volume of blood, its rate of flow, and its pressure. The mechanism of action of these factors is poorly understood.

The point to stress here is that only in a few instances, most strikingly in the dorsal segmental branches of the aorta, do definite vessels first appear as preorganized single tubes. Larger vessels come into existence secondarily within a provisional, primitive capillary plexus that is developed in the embryonal anlage. Consequently, the vascularity of a tissue is determined by conditions that induce growth of capillaries within the anlage and not by the possession of larger vessels initially. Indeed, large vessels emerge late as service pipes to supply an expanding capillary bed (*2*). The spreading of capillaries from primary vascular centers into areas not yet vascularized proceeds at an uneven pace. For instance, the neural tube attracts vessels at an early developmental stage, with the lateral part of the tube being vascularized earlier than the roof or the floor. The mesenchymal condensation that represents premuscle is vascularized rather late, and tissues like cartilage and cornea remain permanently avascular. Thus the angiotaxic influence originally exerted by the cell population of the anlage influences the size of the primitive capillary network that, in turn, determines the size of the fully developed vascular bed of an organ. A similar set of events appears to occur in the vascularization of neoplastic tissues.

VASCULARITY OF TUMORS

The observation that extensive angiogenesis occurs around tumor transplants was made several decades ago (*6–11*). One of the first questions generated by this observation concerned the pattern of the newly formed vessels. Despite some peculiarity of architecture that could be related to certain types of tumor cells (*12, 13*), the work in this area failed to establish any general rule that linked patterns of vascularity to neoplastic transformation. Quantitation of vascular spaces, however, revealed that, at least in solid tumors of rodents, the size of the vascular network per unit volume remained relatively constant for each tumor type (*14*). For instance, in a group of hepatomas produced by a variety of chemical carcinogens, the vascular space measured by high-molecular-weight dextran varied from 4.5 to 12.4% of the tumor weight. Each hepatoma, however, maintained its characteristic vascular space over several transplant generations. When the cell population of 2 hepatomas was changed by selective

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[2] Received April 10, 1978.

Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

transplantation, the vascular space of the solid tumors derived from each line also changed (*14*). This type of evidence sustains the opinion that a neoplastic cell population "controls" the size of its vascular network.

Contrary to the situation in the embryo, however, the functional efficiency of the newly formed vascular network appears to diminish as the tumor grows. Vogel (*15*) observed that the vascular space of a mouse mammary adenocarcinoma remained constant as the tumor grew from 5 mg to 2.7 g; however, the length of capillaries and sinusoidal channels decreased from 820 to 47 mm/mm^3 of tissue, the lumen increased in size, and the surface area available for metabolic exchanges decreased from 34 to 7 mm^2/mm^3 of tissue.

The fluid balance within the tumor is also a major factor that influences the efficiency of the vascular network. A lymphatic network is not anatomically evident in tumors, but a fluid transfer within the interstitial compartment compensates for this deficiency. In rat carcinomas, this transfer corresponded to 5-10% of the total fluid passing through the vascular network (*16*). The hydrostatic pressure produced under these circumstances reaches values of 8-30 cm H_2O in contrast to a pressure of almost zero in the subcutaneous tissue into which the tumor was transplanted (*16, 17*). This high interstitial pressure may be responsible for the enlargement of the interstitial space, as is commonly found in neoplastic tissues (*18*), and for the compression of vessels that is usually considered one of the causes of tumor necrosis (*19*).

Two events are known to contribute most to the deterioration of the vascular network of the growing tumor: the increased pressure of the interstitial fluid, as previously mentioned, and the turnover time of capillary endothelium. Tannock (*20*) and Tannock and Hayashi (*21*) observed in C3H/He mice that this turnover time was about 50 hours, whether measured in a mammary carcinoma or in the fractured femur. Because the doubling time of many neoplastic cells exceeds that of the endothelium, a deficiency of capillary formation may be expected in the growing tumor. Cliff (*22*) determined that the mean growth of blood vessel sprouts in the granulation tissue of wounds was 5.0±2.0 μ/hour and the average migration velocity of the endothelial cells was 4.1 μ/hour for cells of an average size of 32.5 μ in length and 7.7 μ in width. To my knowledge, these kinds of measurements have not been made for capillaries in tumors, although active migration of endothelium can be seen, for instance, in capillary sprouts growing toward corneal implants of tumors (*23*).

ANGIOGENESIS AND TUMOR GROWTH

The experiment that dramatized the interdependence between angiogenesis and tumor growth was that of Folkman et al. (*24*). Isolated thyroid glands from dogs or rabbits were perfused with tissue culture medium, and melanoma cells from mice and rats were added to the fluid. A large number of neoplastic foci appeared within the thyroid gland but none grew beyond a 1- to 2-mm diameter, despite the fact that perfusion continued for many days. The cells were alive and produced rapidly growing nodes when retransplanted into the original host but were unable to do so in the perfused organ. There was no indication that immunologic events caused this growth arrest. However, alteration of the vascular endothelium was consistently observed in the perfused thyroid glands, just as is seen in other perfused organ systems. Folkman suspected that the lack of endothelial proliferation prevented new formation of capillaries and, consequently, tumor growth.

In support of this hypothesis, Folkman and coworkers initiated an extensive analysis of the conditions under which angiogenesis by tumor implants occurred (*25*). They demonstrated convincingly that a clinically evident tumor was formed only when the host tissues provided the vessels (*26, 27*). When neoplastic cells were present, the surrounding endothelium started to proliferate and the thymidine labeling index increased from 0.7 to 8% (*23, 28*). Implants in avascular areas, such as the anterior chamber of the eye or the vitreous, survived but failed to produce tumors unless the tumor fragments could adhere to a surface with vessels approximately 2 mm distant (*27*).

The probability that neoplastic cells produce an angiogenic factor was sustained by the observation of a vasoproliferative effect produced by melanoma or choriocarcinoma cells separated by a Millipore filter from the cheek pouch stroma of hamsters (*29, 30*). In our laboratory (*31*), we use chambers with walls constituted by Millipore filters to sample in vivo the interstitial fluid of a variety of tumors; this cell-free fluid has a strong angiogenic capacity (unpublished observations).

The ability to induce angiogenesis is not peculiar to neoplastic cells. Angiogenesis has also been induced by lymphocytes of spleen ~~and lymph nodes, by~~ hydrocortisone-resistant thymocytes from mice (*32, 33*), by macrophages obtained from the peritoneal cavity of guinea pigs or mice that had been given injections of, respectively, paraffin oil or thioglycollate (*34*), and by testicular grafts of 1-day-old mice (*35*). Moreover, vascular proliferation usually follows leukocyte invasion of the cornea (*36*).

The nature of the angiogenesis factor released into the interstitial fluid is still unknown (*37*). Tuan and co-workers (*38*) isolated from nuclei of cells from Walker rat carcinoma 256 nonhistone proteins that were mitogenic to endothelial cells and caused blood vessel proliferation when implanted in the cornea. Also from Walker carcinoma 256, Kumar (personal communication) isolated two fractions with angiogenic activity and produced an antiserum able to nullify the angiogenic capacity of Walker carcinoma extracts. Angiogenic activity has also been found in the lyophilized medium of tumor cell cultures (*39*). Purification and characterization of this material are in progress (*40*).

The lack of a better characterization of the angiogenesis factor(s) severely limits studies on the correlation between neoplastic transformation and angiogenic capacity of cells. Four methods are in general use to assess angiogenic capacity: the subcutaneous dorsal pouch in the rat, the corneal pocket and iris in the rabbit or guinea pig, and the chorioallantoic membrane of the chick embryo (*8, 34, 40–43*). By all four methods one observes new formation of vessels by direct inspection of an area that is either devoid of vessels initially or has a vascular pattern clearly distinguishable from the newly formed capillaries. Recognition of angiogenesis is facilitated by the more rapid leakage of fluorescein into newly forming capillaries (*41*). All of these methods, however, permit only a qualitative evaluation of angiogenesis. Only the intradermal procedure followed by Sidky and Auerbach (*32*) offers a quantitative approach based on the measurement of induced vascular reticulation. This approach, however, has limited applicability. Cultured endothelium has long been considered a good candidate for a quantitative test of angiogenic capacity. Although promising attempts have been made (*44*), I am not aware of any successful procedure for routine testing.

Another limitation imposed by the lack of characterization of the angiogenesis factor(s) concerns the distinction between angiogenesis observed in inflammatory granulomas and in tumors. Operationally, however, the distinction can be obtained by treatment with corticosteroids, which inhibit the inflammatory process but not tumor angiogenesis.

ANTIANGIOGENESIS AND AMPLIFICATION OF THE ANGIOGENIC RESPONSE

Suppression of angiogenesis should deprive the neoplastic cell population of the possibility of forming a clinically evident tumor (*45*). This was an obvious conclusion derived from the work on angiogenesis mentioned earlier. The clue to finding antiangiogenesis material was provided by the observation that, in the chick chorioallantoic membrane preparation, hyaline cartilage was not penetrated by vascularized mesenchyme whereas virtually any other tissue was penetrated (*46*). The resistance to penetration was not significantly altered by devitalization but was sharply diminished when the cartilage was extracted with guanidine HCl (*47*). Indeed, scapular cartilage of neonatal New Zealand White rabbits blocked angiogenesis when interposed in the corneal assay between a tumor transplant and the limbal plexus (*48, 49*).

A partially purified fraction of the guanidine HCl extract was obtained by affinity chromatography with trypsin-Sepharose. On acrylamide gel electrophoresis most of the antiangiogenesis activity was localized in a band with a molecular weight between 17,800 and 14,400 (*50*). The extraction procedure was similar to that used to obtain collagenase inhibitor from bovine cartilage (*51*); the fraction with antiangiogenesis activity strongly inhibited protease activity (*50*). Proteolytic enzymes are produced by many transformed cells and by a few normal cell lines and tissues (*52, 53*). Substances that act on cells as promoters of their neoplastic transformation are also able to induce appearance of proteases (*54*). The relationships among acquisition of angiogenic capacity, promotion of neoplastic transformation, appearance of protease activity, and reversion of carcinogenic promotion are still unclear, in particular the role of protease inhibitors in the angiogenesis process.

The work of Sidky and Auerbach (*32*) on the vascular response induced in mouse skin by foreign lymphocytes raises the possibility of a lymphocyte-mediated influence on angiogenesis. Operationally, tumor-induced angiogenesis and lymphocyte-mediated angiogenesis are indistinguishable. The vascular response could be elicited directly by a factor produced by the neoplastic cells (*35*) or it could be mediated by lymphocytes or macrophages, possibly by recognition of tumor antigens.

ANGIOGENESIS AND NEOPLASTIC TRANSFORMATION

Because neoplastic tissue manifests an angiogenic capacity almost consistently whereas normal tissue does so only exceptionally, appearance of active angiogenesis should occur at a certain moment in the process of neoplastic transformation. Possibly, angiogenic capacity is acquired before the cell population has constituted a clinically evident tumor. Thus detection of a sharp increment of angiogenic capacity in a tissue may reveal neoplastic progression.

Mammary tissue has, thus far, provided the best support for this hypothesis. Mouse mammary gland often shows hyperplastic regions as part of the aging process, and most mammary carcinomas have been shown to originate from these areas (*55*). These hyperplastic nodules are useful as experimental tools because some of them can be grown in the mammary fat pad, where they either remain unchanged for months or originate a carcinoma. Medina (*56*) developed two lines of these transplantable hyperplastic tissues: One line (D_1) produces tumors in less than 10% of the transplants, but the other line (D_2) produces tumors in more than 40% of the transplants. When tested for angiogenic capacity, the hyperplastic nodules selected at random showed a response in 30% of the samples. Approximately the same frequency of angiogenesis was observed in the D_1-line having a low tumor potential, but 80% of D_2-transplants showed a strong angiogenic response long before any morphologic sign of neoplasia was visible (*57*). In the rat, mammary hyperplastic tissue that has acquired the property of continuous transplantability also showed a sharp increase in the frequency of angiogenic response (unpublished observations).

The human mammary gland also has a high frequency of hyperplastic regions (*58*), and 28% of transplantable hyperplastic nodules were recently found to possess angiogenic capacity (*59*). The possible correlation between frequency of neoplastic transformation in human mammary parenchyma rich in hyperplastic regions and high frequency of angiogenesis has yet to be studied. Because approximately 70% of the biopsy specimens from "lumps" in the breast are diagnosed as "benign" lesions, often rich in hyperplastic epithelium, the angiogenic response of these lesions might be useful as a marker of neoplastic progression and possibly as a tool to identify women at high risk of developing breast cancer. In practical terms, the high cost and the qualitative nature of the angiogenesis assay have limited its clinical utilization. Expanded knowledge about angiogenesis and progress in finding a satisfactory quantitation of the process may help in tumor prophylaxis.

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GUEST EDITORIAL——by James A. Miller and Elizabeth C. Miller

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Chemical Carcinogenesis: Mechanisms and Approaches to Its Control[1]

THE HISTORY of cancer research shows that the discoveries in man of carcinogenesis by various chemicals preceded studies of these substances in experimental animals. Subsequent laboratory investigations then led to the discovery of the large variety and number of experimental chemical carcinogens. Today the pathways from epidemiological studies to tests in animals and from these tests back to man are being traveled with increasing frequency in attempts to determine and control the chemical, viral, and physical carcinogens in man's external and internal environments.

From these studies, particularly those in industrial environments, a number of compounds and mixtures of chemicals are now generally recognized as carcinogenic in the human (*1–3*). The specific carcinogenic components of various tars, soots, and oils, cigarette smoke, and betel nut for the human have not been elucidated. The better defined carcinogenic chemicals for man include various aromatic amines and related compounds (2-naphthylamine, benzidine, 4-aminobiphenyl, auramine, and magenta), *N*,*N*-bis(2-chloroethyl)-2-naphthylamine, bis(2-chloroethyl)sulfide, asbestos, and certain nickel- and chromium-containing compounds. Many other chemical carcinogens for man surely exist, and a number of chemicals in the environment are on the suspect list. Thus, arsenic compounds have long been suspected of being carcinogenic for human skin, and recent epidemiological evidence also implicates these compounds in the etiology of some respiratory cancers (*2*, *4*). Prolonged excessive use of phenacetin by certain patients appears to have induced kidney tumors (*5*). Neither of these situations is uncomplicated, and so far these agents, unlike those mentioned above, have failed to produce tumors in experimental animals. The aflatoxins and the secondary nitrosamines are under high suspicion as human carcinogens, since they are present in specific human environments and since they are potent carcinogens in many species (*6–9*.)

Modern epidemiological studies of many countries and on migrant groups suggest strongly that several important human cancers have significant environmental factors in their etiology (*10–12*), and our modern medical and chemical age has provided an increased lifespan for the expression of the carcinogenic potentials of these factors in susceptible humans. Therefore, it seems reasonable to expect that lifetime exposures to a variety of environmental chemicals, both man-made and of natural occurrence, are determining factors in a significant and possibly high percentage of human cancer. The determination and control of that fraction of human cancer in which chemical carcinogens play an important role must involve

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Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors will welcome suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

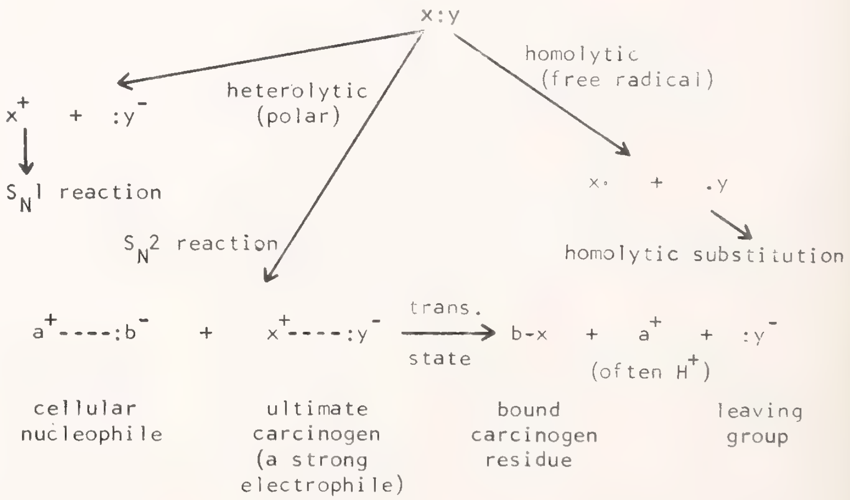
oncologists in many disciplines. Our intent here is to present a broad view of the metabolic activation of chemical carcinogens, the possible mechanisms of action of these agents, and the implications of some current data for approaches to the control of chemical carcinogenesis. Many of these concepts and data have been reviewed previously in more detail by the authors (*1*, *13*, *14*) and other workers cited below.

Metabolic Activation and Reactivity of Chemical Carcinogens

While the chemical carcinogens comprise a wide variety of structures, this variety is not so broad as to support the suspicion, sometimes expressed, that any chemical given chronically at high dosage will induce cancer in some animals. The data do not support this idea. A good example is the recent National Cancer Institute study of 120 pesticides of very varied structural types in two sensitive strains of mice (*15*). Only 10% of these compounds, which were selected for their toxicity as pesticides, showed carcinogenic activity in lifetime tests at high dose levels.

While no common structure is evident among the various chemical carcinogens, it now appears that the great majority, and perhaps all, of the chemical carcinogens are similar to one another in the gross electronic nature of their ultimate reactive forms. Thus, the reactive forms of chemical carcinogens all appear to be strong electrophiles (*i.e.*, molecules with electron-deficient atoms). The reactions of these electrophiles in cells involve nucleophilic molecules containing electron-rich atoms, and the general situations for the reactive electrophile x:y are given in text-figure 1. Each class of bond-breaking, heterolytic and homolytic, yields electron-deficient or electrophilic species. Homolytic cleavage yields free radicals. Heterolytic cleavage can yield positive ions which then react with nucleophiles (S_N1 reaction). Heterolytic cleavage can also occur at the same time that an electrophile (x:y) reacts with a nucleophile (a:b) in an S_N2 (shorthand for substitution, nucleophilic, bimolecular) reaction. In this type of reaction the bond-breaking and bond-making occur simultaneously in a transition state to yield the substituted molecule b:x and the loss of a^+ and $:y^-$. S_N2 reactions or reactions of a type intermediate between S_N1 and S_N2 appear to predominate over pure S_N1 or free radical reactions with the reactive forms of chemical carcinogens (*16*). The S_N2 reaction is detailed at the bottom of text-figure 1.

The carcinogenic alkylating agents were early recognized as compounds which are already in their final reactive forms and which are electrophilic reactants. They react primarily in the S_N2 manner, and their products *in vivo* are the same as those formed *in vitro* in nonenzymatic reactions with tissue components [*see* (*1*)]. Most chemical carcinogens, however, require conversion *in vivo* to the reactive electrophilic x:y state. It is now recognized that metabolic reactions leading to the active x:y state and other metabolic reactions leading to deactivation of x:y and its precursors



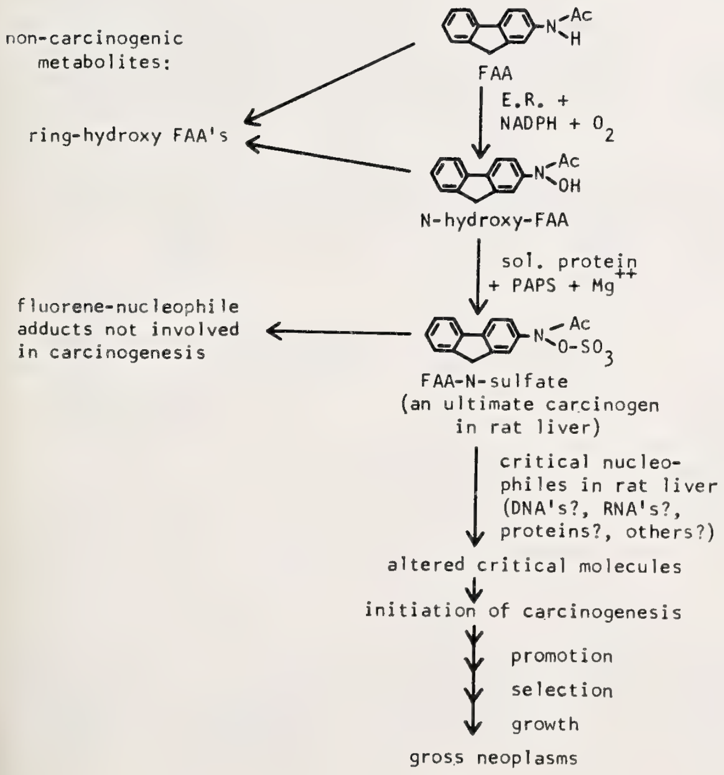
TEXT-FIGURE 1.—Bond-breaking mechanisms and substitution reactions.

account for many tissue and species differences in carcinogenic activity which have long been noted with chemical carcinogens.

Thus, we have the general situation in which the parent unreactive carcinogen or precarcinogen is converted into another molecule that, though unreactive itself, is closer to the final reactive or ultimate carcinogenic form than the precarcinogen. This intermediate has been termed a proximate carcinogen, and it is converted, possibly via other proximate carcinogens, to an ultimate carcinogen which is a reactive electrophile that attacks tissue nucleophiles. Much of the dose of carcinogen may be dissipated in deactivation reactions at each level. However, once enough of the ultimate carcinogenic form has reacted with critical nucleophiles in cells for a long enough time, the chemical agent is no longer needed for the carcinogenic process to continue to the formation of gross cancers.

A specific example of these reactions is shown in text-figure 2 for carcinogenesis in the male rat liver by *N*-2-fluorenylacetamide (FAA) (*1*, *13*, *17*). The first activation step is an enzymatic oxidation in the endoplasmic reticulum where an oxygen molecule is cleaved to form a strongly electrophilic oxygen atom that attacks FAA to yield *N*-hydroxy-FAA. This proximate carcinogen—with greater activity in a wider range of tissues than FAA—is still a nucleophile and is not reactive with tissue components. The soluble fraction of male rat liver contains sulfotransferase(s) that utilize the active form of sulfate, 3′-phosphoadenosyl-5′-phosphosulfate (PAPS), to generate FAA-*N*-sulfate, which is a highly reactive electrophile that attacks nucleophilic tissue components. A variety of evidence strongly points to this electrophile as a major ultimate carcinogen of FAA in male rat liver (*1*, *17*, *18*). The natures of the ultimate reactive and carcinogenic forms of FAA at other sites of tumor formation in the rat are unknown.

Species and sex differences are very evident in these activation steps. The *N*-hydroxylation step for FAA appears to be lacking in guinea pig liver, which is resistant to FAA. While *N*-hydroxylation is extensive in the female rat (Charles River CD) liver and in the male rabbit, mouse, and hamster livers, the livers of these animals have only low levels of sulfotransferase activity for *N*-hydroxy-FAA and are thus highly resistant to hepatocarcinogenesis by *N*-hydroxy-FAA. The ring-hydroxy-FAA's and low molecular weight nucleophile adducts appear to be products of deactivation and


non-carcinogenic metabolites:
ring-hydroxy FAA's
FAA
E.R. + NADPH + O_2
N-hydroxy-FAA
sol. protein + PAPS + Mg^{++}
fluorene-nucleophile adducts not involved in carcinogenesis
FAA-N-sulfate (an ultimate carcinogen in rat liver)
critical nucleophiles in rat liver (DNA's?, RNA's?, proteins?, others?)
altered critical molecules
initiation of carcinogenesis
promotion
selection
growth
gross neoplasms
Ac
H
OH
$O-SO_3$
N

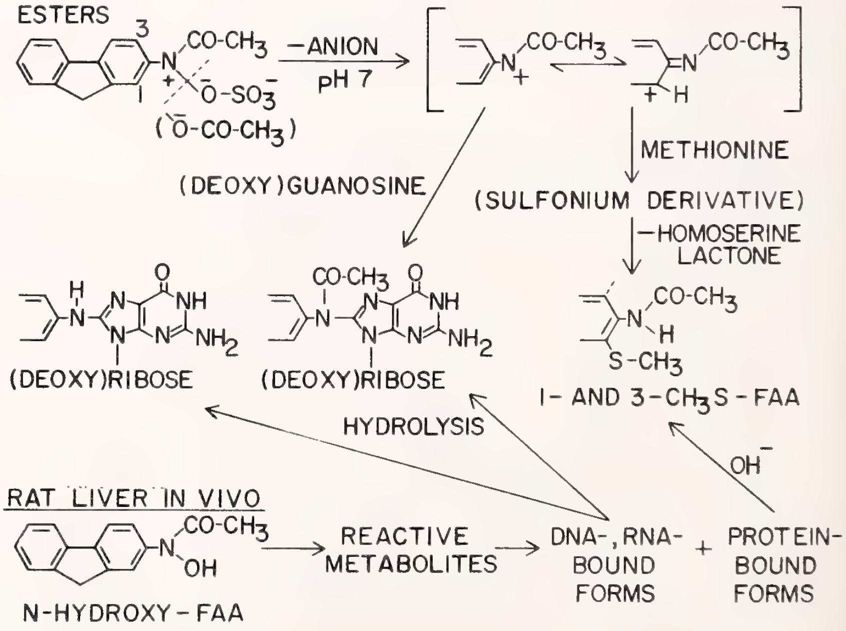
TEXT-FIGURE 2.—Major routes of activation and deactivation of *N*-2-fluorenylacetamide (FAA) in the male rat liver.

their rates of formation are thus among the factors that determine the dose of FAA needed for carcinogenesis.

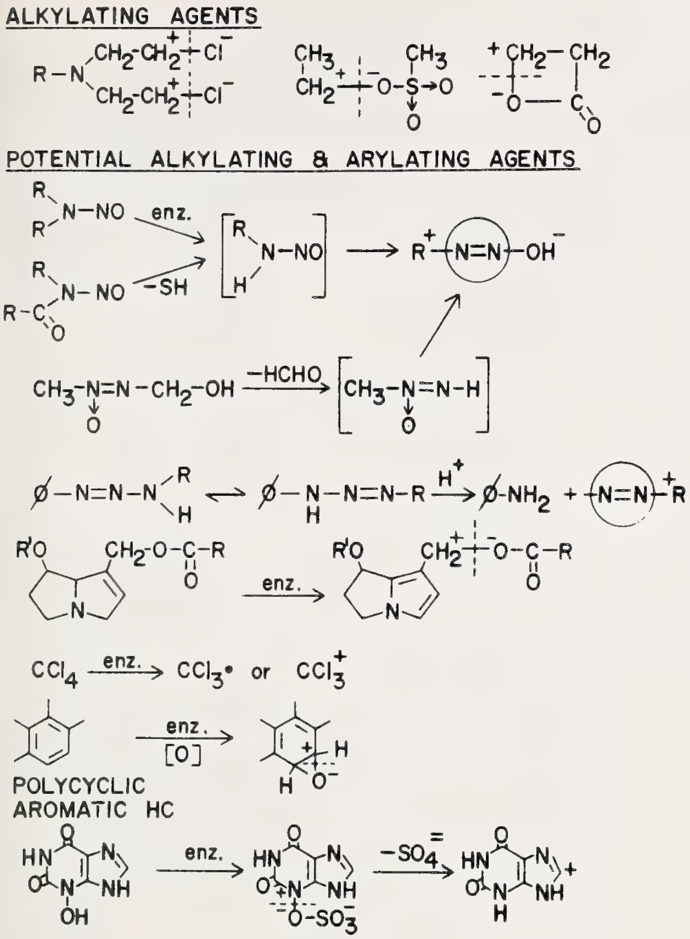
Two of the nucleophiles that can be attacked by FAA-*N*-sulfate are shown in text-figure 3. One is methionine whose adducts readily decompose to yield 1- and 3-methylmercapto-FAA. These methylmercapto derivatives are also obtained from the liver protein of rats fed *N*-hydroxy-FAA. In addition to the possible effects of the fluorene substituent on protein function, the decomposition of the methionyl derivatives can result in cleavage of methionyl peptide bonds and consequent modification of the reacted protein (*1*). The esters of *N*-hydroxy-FAA also react with nucleophiles in nucleic acids and, in bacterial-transforming DNA, these reactions result in both inactivating and mutagenic effects (*19*). The reaction of FAA-*N*-sulfate with guanine residues, the major site of attack in nucleic acids, is shown in text-figure 3. The same derivatives have been obtained on degradation of the liver DNA and RNA from rats given injections of *N*-hydroxy-FAA. The corresponding nonacetylated 2-fluorenamine derivatives are also obtained from the liver nucleic acids, but the evidence suggests that the acetylated derivatives are more closely related to hepatocarcinogenesis (*1*, *20*).

There is now considerable information on the formation of electrophilic derivatives as the ultimate reactive forms of a wide variety of chemical carcinogens, and we have discussed these data in more detail elsewhere (*1*, *13*, *14*). Illustrative examples are shown in text-figure 4. Thus, the carcinogenic alkylating agents, such as the nitrogen mustards, alkyl sulfonate, and strained lactone shown in the figure, are reactive as administered. The dialkylnitrosamines, alkylnitrosamides, methylazoxymethanol (the proximate carcinogen from the naturally occurring carcinogen cycasin), and the alkyl aryl triazenes all yield alkylating species via enzymatic or nonenzymatic reactions *in vivo*. The toxic pyrrolizidine alkaloids and carbon tetrachloride are similarly metabolized to electrophilic species *in vivo*. Epoxides of some carcinogenic polycyclic aromatic hydrocarbons are more active than the parent hydrocarbons for the transformation of cells *in vitro* (*21*); these reactive electrophiles are also apparently formed *in vivo* and *in vitro* (*22*, *23*). Finally, the highly electrophilic sulfuric acid ester of the carcinogen 3-hydroxyxanthine can be formed by liver preparations, and the excretion of 8-chloro- and 8-methylmercaptoxanthine by rats administered 3-hydroxyxanthine indicates that esters of this type are formed *in vivo* (*24*, *25*).

The nucleophilic sites in cells known to be attacked *in vivo* by electrophilic reactants derived from chemical carcinogens include a variety of amino acid residues in proteins and bases in



TEXT-FIGURE 3.—Reactions of esters of *N*-hydroxy-FAA with methionine and guanine derivatives and the formation of the same products on degradation of liver protein and nucleic acids from rats administered *N*-hydroxy-FAA.



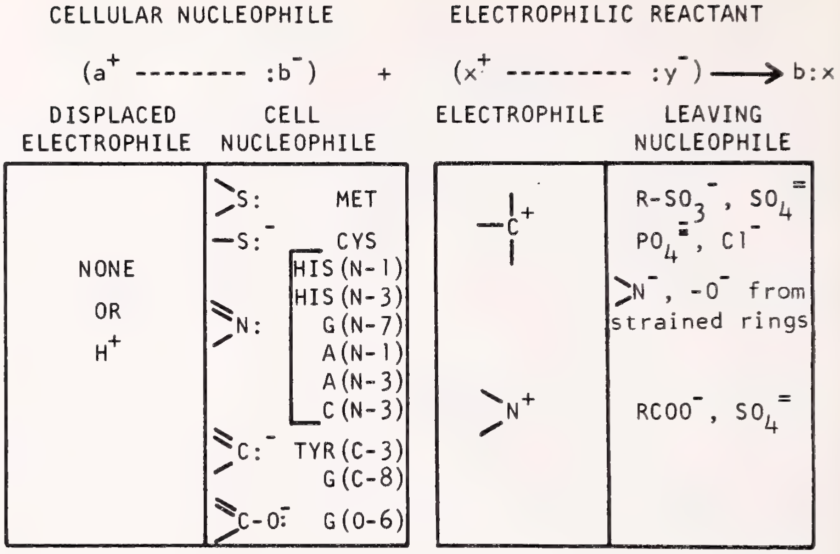
TEXT-FIGURE 4.—Probable ultimate reactive and carcinogenic metabolites of certain carcinogens.

nucleic acids (text-fig. 5). The degree to which these nucleophilic sites are attacked *in vivo* varies greatly and depends on both electronic and steric factors. Other nucleophilic sites of attack must also be available in cells. It is of interest that proteins and nucleic acids contain many more nucleophilic than electrophilic sites.

Relationship Between Carcinogenic and Mutagenic Activities of Chemicals

Carcinogenesis and mutagenesis are grossly alike in that each process leads to heritable changes in phenotype. Likewise, chemical carcinogens and chemical mutagens have much in common in that electrophilic reactants are the active forms of most, and possibly all, carcinogens and of many, but not all, chemical mutagens (*26*). The major exceptions among the chemical mutagens appear to be in the frame-shift-inducing mutagens and the mutagenic base analogs; neither of these two classes of mutagens has been adequately tested for carcinogenic activity.

The critical cellular target of chemical mutagens is DNA, while the critical target(s) of chemical carcinogens has not been defined. However, the electrophilic nature of the reactive forms of most carcinogens and most mutagens and the fact that DNA's, RNA's, and proteins are all targets of electrophilic reactants make clear that no gross correlation between carcinogenicity and mutagenicity, no matter how good, could establish carcinogenicity as a mutagenic event. The best conclusions that can be drawn from the present data are that many, perhaps all, chemical carcinogens are potential mutagens and that many, but possibly not all, chemical mutagens are potential carcinogens (*26*). As will be emphasized later, these conclusions appear to have some practical value, even though they give no information as to the mechanisms of chemical carcinogenesis.



TEXT-FIGURE 5.—Cellular nucleophiles known to be attacked by electrophilic derivatives of carcinogens.

Possible Mechanisms of Chemical Carcinogenesis

Since the active forms of chemical carcinogens can and do react with each class of informational macromolecule in cells, it is necessary to consider both genetic and epigenetic mechanisms of chemical carcinogenesis. The first genetic mechanism noted in table 1 is the somatic mutation concept in which the copying of a chemically changed DNA could lead to alterations, deletions, or rearrangements of the DNA nucleotide sequence that would be perpetuated with permanent changes in growth control. The second mechanism, the fixation in DNA of alterations which occurred in RNA, is based on the new phenomenon of reverse transcription (*27–29*). The third mechanism is based on the observations of Speyer and associates (*30*) that certain mutants of bacteriophage T_4 have a high spontaneous mutation frequency because of the poor accuracy with which their DNA polymerase copies the phage DNA. Chemical alteration of only a few DNA polymerase molecules might be sufficient to introduce critical mutations into cellular DNA favoring the eventual emergence of neoplastic cells.

The reactions of chemical carcinogens with proteins and RNA's lead to considerations of epigenetic mechanisms (table 1). The first epigenetic mechanism listed is based on the currently popular model of cellular differentiation. The variety of expressions of embryonic antigens and enzymes in chemically induced tumors, which have been reported in the past few years, could be examples of effects of chemical carcinogens on gene read-out. These changes in transcription could also include derepression of part or all of integrated tumor virus genomes or of the oncogenes of Huebner and Todaro (*31*).

Epigenetic mechanisms can also be envisaged that would permit the preferential proliferation of previously existing neoplastic or preneoplastic cells. For instance, carcinogen-induced changes in the immunological capacity or hormonal balance of the host could give proliferative advantages to such cells.

TABLE 1.—Possible mechanisms of chemical carcinogenesis

I. Genetic mechanisms—heritable changes in DNA genome via:
    1. Direct modification of existing DNA.
    2. Modification of RNA which is subsequently transcribed into DNA that becomes integrated into host DNA.
    3. Alterations which decrease, at least temporarily, the fidelity of copying of DNA.
II. Epigenetic mechanisms—nongenomic changes leading to:
    1. Quasi-permanent changes in the transcription of DNA (including integrated virus genomes and oncogenes).
    2. The preferential proliferation of previously existing preneoplastic or neoplastic cells.

Approaches to Prevention or Inhibition of Chemical Carcinogenesis in Man

Incomplete as the present knowledge of chemical carcinogenesis is, it is important to consider what kinds of controls might be used to prevent or delay chemical carcinogenesis in man. The most obvious means of control is through reduced exposure to carcinogens (table 2). The collection of information for this purpose obviously needs much more emphasis, even though the cigarette-lung cancer problem shows how difficult it may be to achieve a reduction in tumor incidence when sound preventive information is at hand. More penetrating epidemiological studies are needed, and these should go hand in hand with experimental studies to identify potential carcinogenic hazards in man.

In view of the varied ways in which chemical carcinogens are activated in cells, broad-spectrum chemical or physical tests to identify chemical carcinogens are not likely to be found. So far, no broad-spectrum biological tests for chemical carcinogens have appeared that do not depend on tumor formation. Thus, it appears that the lifetime or 2-year carcinogenicity assays in rodents treated with high levels of the test compounds will be required for the indefinite future, despite the relative insensitivity and other deficiencies of these assays. However, there are good prospects for improving the reliability with which the results of the rodent carcinogenicity assays can be used for assessing potential hazard for man. For this it may be possible to develop important tools from mutagenicity assays and cell transformation assays which utilize the host-mediation principle of Legator and Malling (*32*). Thus, by assessing the mutation frequency of indicator bacteria or other microorganisms inoculated into experimental animals receiving the test compound or added with the test compound to cell cultures or tissue preparations from experimental animals and humans, the detection of those compounds which are converted into electrophilic reactants in experimental and human tissues should be facilitated. Similar assays for cell transformation or carcinogenesis *in vitro* may also be possible. These host-mediated assays might help to reveal instances in which the results of lifetime carcinogenicity assays in rodents might be suspected of giving false-positive or false-negative results with respect to the probable hazard for man. The host-mediated mutagenicity assays would also be helpful in setting up priorities for the carcinogenicity testing of the hundreds of compounds on which tests are now needed or will be needed in the future. Some assistance in setting priorities for carcinogenicity assays may also be gained from a knowledge of the structures and metabolism of suspect compounds. Critical transient metabolites such as strong electrophiles are not easily isolated, but the detection of covalent binding of labeled foreign molecules to nucleic acids and proteins in tissues could be used to indicate the probable formation *in vivo* of strongly electrophilic metabolites.

TABLE 2.—Approaches to the inhibition of chemical carcinogenesis in the human through reduction of exposure to carcinogens

I. Further epidemiological studies to recognize carcinogenic hazards to which man is or has been exposed.
II. Further studies to recognize potential carcinogenic hazards for man, especially via:
    a. Carcinogenicity assays.
    b. Mutagenicity assays (especially host-mediated).
    c. Cell transformation assays *in vitro* (especially host-mediated).
    d. Consideration of structure and metabolism of known and possible carcinogens.
III. Further development of legislation and analytical methods for protection of the public. Consideration must be given, where necessary, whether the possible carcinogenic hazard is less critical than the risk due to lack of use of a potential carcinogen.

Further protective legislation may be needed. For instance, the Delaney clause in the U.S. Food, Drug, and Cosmetic Act, which prohibits the use of carcinogenic substances as food additives, may need to be broadened to include other parts of our environment. Analytical methods, such as urinary assays for metabolites of the aflatoxins, must be devised to provide measures of total exposure to certain potential human carcinogens. Lastly, judicious consideration needs to be accorded the uses of substances which, though they may show carcinogenic activity in certain experimental situations, also have important beneficial effects. The probable low risk to benefit ratio in the use of DDT in malaria control is a case in point.

Metabolic attacks of the host on chemical carcinogens and their metabolites provide, at least theoretically, another avenue of control (table 3). To the extent that one could increase the de-

TABLE 3.—Approaches to the inhibition of chemical carcinogenesis through alteration of metabolism of the carcinogen

I. Inhibition through increased metabolism to inactive derivatives (*33, 34*).
  Example: Increased oxidase levels in hepatic endoplasmic reticulum lead to inhibition of:
  a. FAA-induced liver and ear duct gland carcinogenesis (rat).
  b. Polycyclic hydrocarbon-induced mammary gland (rat) and lung carcinogenesis (mouse).

II. Inhibition through decreased metabolism to ultimate carcinogens
  Examples:
  a. Inhibition of skin tumorigenesis in mice by simultaneous application of a polycyclic hydrocarbon and an inhibitor of its oxidative activation (*35*).
  b. Inhibition of FAA-induced hepatic carcinogenesis by depletion of sulfate pool (*18*).

activation and restrain the activation of chemical carcinogens, the tumor response should be reduced. Some experimental models of these processes are mentioned in the table, but it is not easy to see how mechanisms of these types could be adapted for practical use in the human situation. Such modifying situations may occur in nature. Thus, as shown recently by Wattenberg (*33*), certain vegetables contain substances that induce very large increases in the aryl hydrocarbon hydroxylase activity in rat intestine and lung. At best, possible protective mechanisms based on increased levels of the deactivating enzymes would be complicated, since closely related enzymes may be involved in both the activation and deactivation of chemical carcinogens; effects opposite to those desired could result.

Finally, the electrophilic nature of the active forms of chemical carcinogens predicts that one could inhibit chemical carcinogenesis by administration of noncritical nucleophiles which would react with these electrophiles. Some preliminary positive data are being obtained in our laboratory with the partial inhibition of hepatic carcinogenesis by FAA and *N*-hydroxy-FAA with excess dietary methionine, cystine, or tryptophan (*36*). Indeed, inhibition of carcinogenesis via trapping of the ultimate carcinogen(s) with noncritical nucleophiles probably occurs to some extent with all chemical carcinogens and may provide a natural protective mechanism, especially with the very small doses of carcinogens we are likely to face in our environment at any one time. The removal of ultimate carcinogenic forms through noncarcinogenic reactions may be an important factor in the determination of threshold doses of carcinogens or changes in the slopes of dose-response curves at low doses of carcinogens.

Conclusion

In this brief survey we have attempted to highlight aspects of chemical carcinogenesis which seem to be major links to future work in the field—especially as it may relate to chemical carcinogenesis as a practical problem for man. If attempts to understand and control chemical carcinogenesis are extended hand in hand with the developing knowledge in cancer epidemiology and viral carcinogenesis, it seems reasonable to expect that control of the occurrence of some important cancers in man may be in sight by the end of this century. Neither the scientific nor lay public should underestimate the probable long lag-time (possibly several decades) between the institution of a successful control measure and noticeable reductions in the incidence of a human cancer. Hopefully, this may not always be the case. Thus, if a compound involved in the etiology of a human cancer is a promoter or cocarcinogen, its removal from the environment may result in noticeable reductions in cancer incidence in a shorter period. In any event, progress in the prevention and treatment of cancer in man is assured to the extent that dedicated and inspired efforts in the oncological sciences are assured of long-term support.

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EDITORIAL——**by Mearl F. Stanton, *M.D.***

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Fiber Carcinogenesis: Is Asbestos the Only Hazard?

DATA FROM several laboratories as well as my own indicate that asbestos causes cancer, not because it is asbestos, but because of the unique physical structure it can attain (*1*). Asbestos causes cancer when it separates into fibers with exceptionally small diameters that retain substantial lengths. This concept may explain many differences in cancer risk during various occupational exposures to asbestos. It is particularly supported by the correlation shown by Timbrell et al. between differing mesothelioma risk and differing fiber diameters of amphibole asbestos from the Northwest Cape and Transvaal mining regions of South Africa (*1*). However, the basis for this premise is evidence gained from our work and that of others in which various types of fibers were implanted in the pleura of rats and yielded sarcomas essentially the same as the mesotheliomas induced by asbestos in man (*1–3*). This procedure circumvents the variables dependent on the ability of a fiber to reach the target organ and thus becomes an initial step to understanding mechanisms of cancer induction by fibers. These studies indicate that neither the hydrocarbons and metal-mineral complexes naturally associated with asbestos nor those associated with the processing of asbestos are sufficient to account for asbestos carcinogenicity. Further, the studies indicate little difference in the carcinogenicity of types of asbestos that vary widely in physicochemical structure, if they are milled to similar ranges of size.

The most convincing evidence of a structural relationship to carcinogenicity is that fine, long fibers of durable materials completely unrelated to asbestos, i.e., glass and aluminum oxide, are similarly carcinogenic in the pleura of the rat. On the other hand, nonfibrous particles of asbestos, glass, or aluminum oxide, as well as exceptionally short fibers of these materials, are far less carcinogenic in the pleura of the rat. In fact, the degree of carcinogenicity of the 3 materials correlates with the dimensional range of the fibers implanted. Because fibers in the samples vary widely in size, carcinogenicity cannot be confined to an absolute dimensional range. But a conservative estimate indicates that, in the pleura of the rat, durable fibers $<3\mu$ in diameter and $>20\mu$ in length are far more carcinogenic than either fibers of various lengths with diameters $>3\mu$ or fibers with diameters $<3\mu$ and lengths $<20\mu$. Some of our preliminary data indicate that exceptionally long glass fibers with diameters $<0.5\mu$ are highly carcinogenic in the pleura of the rat. We are now testing sized samples of durable fibers of many types to improve our estimates of which dimensions are the most hazardous.

Precise parameters need not be defined to appreciate that carcinogenicity can depend on the structure rather than the physicochemical composition of a particle. The most important corollary to this observation is that any type of durable fiber falling into the broadly defined dimensional range of long, fine fibers may be carcinogenic. Certainly this seems true for the pleura of the rat, and it is unlikely that different mechanisms are operative in man. If we consider the conventional definition of a fiber as any particle with a length >3 times its diameter, minute fibers are abundant in our environment, and the list of fibers used by man increases daily. Durable fibers are used not only as insulation materials and binders of high tensile strength, but also as excellent filters, abrasives, lubricants, emollients, and chemically inert fillers that can be found in everyday household products, e.g., cleansers, paints, cosmetics, and even fabrics, foods, and medications. Furthermore, air and water supplies are frequently contaminated with mineral fibers from natural sources as well as with pollution by man. The proof of man's exposure to fibers lies in the fact that fibers of a variety of types can be isolated from nearly all human lungs.

The question raised is the risk that such fibers may hold currently or with the increasing use of fibers that may occur in the future. A number of observations already made are perhaps relevant to this question (*1–3*); I mention here only a few. The fibrous materials to which we are most commonly exposed consist for the most part of particles too large or too small to be considered potent carcinogens, if the dimensional ranges we have estimated in the rat are used as criteria. For example, most insulation materials in use today, other than asbestos, are composed largely of fibers with diameters $>5\mu$. Erosion and friction wastes of asbestos and other fibrous products are, for the most part, short fibers of submicroscopic size, which in rats seem far less hazardous than fibers in the lowest range of optical visibility. Were it not for these fortunate circumstances, the problem of fiber carcinogenicity might be more obvious today. The most hazardous fibers may be those of exceptional length, with diameters of $<0.5\mu$; such fibers are near the limits of optical visibility and are to some extent in nearly all fibrous materials. Our present systems of assessing carcinogenicity in animals can at best only detect response to large numbers of fibers; therefore, materials containing only a few exceptionally fine fibers could easily escape detection as a hazard in simply designed animal tests. Even with the most sophisticated test, it would be difficult to establish an absolute threshold of safety, since each small exposure possibly adds to the total hazard and represents a finite, albeit small, ultimate risk.

The ultimate risk of fibers is, of course, a complex problem. In addition to the level of exposure and physical properties of fibers, one must take into account factors of inherent resistance, mechanisms of elimination and containment, the properties and opportunities that enable particular types of fibers to reach tissues, and the contributions of other carcinogens such as cigarette smoke. From our experiences with asbestos, it is apparent that fibers can be most subtle carcinogens that deviate from simple dose-response relationships and that require long latent periods before showing carcinogenic activity. For these reasons alone, it would be unwise to ignore a potential problem of greater range than asbestos.

There is perhaps one way to determine the hazards of fibers without waiting the many years necessary for the effects of even massive exposure to become evident. Unlike most carcinogens, fibers that are a threat are sufficiently durable to remain in the tissues from which cancers are derived. Since carcinogenic response can be related to doses of sized fibers in experimental animals, it may be possible to equate the number and size distribution of fibers in human tissues to cancer in man. Although much has been accomplished in assessing large, protein-coated fibers in human lungs, surprisingly little has been done in assessing the size distribution and total quantity of all fibers in human tissues. This would be a tedious job, but it might determine the true significance of fibers as carcinogens in man.

Further, we need clues to the intimate events between cell and fiber that lead to cancer. Not all cells penetrated by fibers die. Conceivably these transfixing fibers could irreversibly alter intracellular replicative processes either directly, through activation of repressed oncogenes, through alteration of cell membranes, or through initiation of a unique, precancerous fibrosis. Short, fine fibers may be less carcinogenic, simply because they are more effectively contained by overt or intracellular phagocytic activity. However, a more complex explanation is suggested by recent studies showing that in vitro growth of cells on minute glass fibers is related to the length of the fiber (*4*). Whatever the mechanism of fiber carcinogenesis is, I believe that the following premise must be taken into account. Durable fibers, perhaps at the extreme ranges of dimension, cause cancer simply because they are fibers and irrespective of their physicochemical nature. Vigilance in detecting the presence of such fibers in the environment and in the tissues of man may well profit us all.

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Molecular Orbital Theory in Carcinogenesis Research

Molecular orbital calculations are a language used to explain a body of chemical data and to predict the ease and course of reactions of chemical compounds. Unfortunately, the use of this language in carcinogenesis research has suffered some abuse by its practitioners and more by those who have recognized that often too much has been claimed for it in attempts to explain the mechanisms of chemical carcinogenesis.

With the development of quantum mechanics, it was immediately apparent that the exact calculations which so accurately explained the properties of a simple species such as H_2^+ could only be approximated in handling larger molecules (*1*). The coming of high-speed computers permitted more sophisticated approximations, but approximations they remained. The most useful approximation for practicing organic chemists was the linear combination of atomic orbitals into molecular orbitals method (LCAO–MO), for this procedure permitted quantitative predictions of chemical behavior for many organic molecules, with a requirement only for desk calculators. The simplest (Hückel) form of the LCAO–MO method could be expanded to cover many other molecules with the use of computers requiring only the simplest type of programming. The ability of the LCAO–MO method to predict chemistry of polycyclic aromatic hydrocarbons is truly remarkable, considering the many simplifying assumptions made for this procedure.

Many of the approximations have been improved by semiempirical methods, i.e., those in which knowledge of the thermochemical or electrochemical properties of compounds is used to modify the calculations for better agreement with experimental observations. Although this may be an unsatisfying approach for those concerned with the niceties of quantum theory, it is a most practical method for constructing a language that will be generally useful for the practicing chemist. Others who preferred to improve on the theory itself chose to observe the extent and direction of deviation between theory and fact and to delete approximations previously made for the sake of convenience. The result has usually been in the same direction as the semiempirical methods.

With the blossoming of systematic carcinogenesis research, particularly with polycyclic hydrocarbons, at the time of development of the MO theory and of a romantic belief among physical scientists that the riddles of biology would yield to the new knowledge of chemistry and physics (*2*), it was only natural that attempts would be made to explain the biological activity of carcinogenic compounds on the basis of predicted chemical behavior in the cell. At this point this desirable merging of chemistry and biology went awry because of two assumptions underlying most attempts to explain carcinogenic activity directly with MO methods: 1) Reactions characteristic of the carcinogen in the artificial systems of the organic chemist will be the controlling reactions biologically; 2) the carcinogen reacts directly with the sensitive target in the cell, and this reaction (repeated, if necessary) is sufficient to produce cancer. It is probably a worthwhile project for a historian of science to determine whether such assumptions were justifiable at the time. With the benefit of 20 years of hindsight, however, we can say that such an approach cannot be justified now. However, chemical carcinogens do undergo chemical processes in the cell and they do react in some form with cell macromolecules. An assumption, which still seems quite justifiable and accepted by many, is that one or more of these reactions is necessary for the carcinogenic process, even if the reaction is not sufficient cause in itself. The events leading to this reaction and other parallel events can be described by MO calculations, at whatever level of sophistication is necessary and available. Let us consider some of these events.

TRANSPORT OF THE CARCINOGEN

Hansch developed the concept that the action of all drugs in a particular class involves interaction with the receptor at roughly the same rate, and that the efficacy of the drug depends on the rate at which it reaches the receptor from the site of administration. This rate, in turn, is proposed to depend on an optimum partition coefficient for the particular system. This basic hypothesis has worked remarkably well and even produced a fairly good correlation between optimum partition and carcinogenic activity for an extended series of amino azo dyes (*3*). Herndon (*4*) later combined a molecular size factor with resonance theory calculations in a regression analysis that appeared to lead to a reasonable ability to predict carcinogenicity from these properties alone. However, a study of his data shows no improvement in reliability over previous approaches that neglect molecular size. Franke and Büchner (*5*) and Franke (*6*) have also suggested that the partition coefficient of a carcinogen may play a major role in its activity. If this property is indeed critical for carcinogenic activity, the ability to predict it reliably by theoretical methods would be desirable. Since partition coefficient is intimately related to the electron distribution within the molecule, such a prediction

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Editor's note: Periodically the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

should be possible. Rogers and Cammarata (7) have shown, with several classes of compounds, that this can indeed be done. Although the procedure requires computer aid, here too the initial program is not excessively difficult. Once developed, the method is capable of generating theoretical parameters for large numbers of compounds with far less effort than would be required to actually make the experimental determination.

METABOLISM OF THE CARCINOGEN

Although it is widely accepted that nonreactive carcinogens must be metabolized to exert their effect, studies of carcinogen metabolism are at a relatively primitive state. Extensive product studies on several aromatic hydrocarbons and a few aromatic amines have been undertaken with the major conclusion being that both classes of compounds are predominantly converted to hydroxylated derivatives by a system requiring NADPH and molecular oxygen. The fact of an intermediate epoxide in some hydrocarbon oxidation has been established, and the oxidation activity has been localized in certain protein fractions. Except for occasional efforts to establish nonenzymatic model oxidizing systems, however, there has been virtually no effort devoted to answering several long-standing questions. Why is a particular set of metabolites produced from a given compound? Why is an alteration in this pattern produced by certain inducing agents? Why do different inducing agents have different effects on metabolism of a given reference substrate? An additional assumption implicit in many attempts to correlate MO parameters with carcinogenicity is that electronic properties of the carcinogen are solely or primarily responsible for the action of the compound. The Hansch analysis does not detract from this position, but rather reinforces it, as demonstrated by the study of Rogers and Cammarata (7). Based on predictions of reactivity, however, such a position in untenable.

Any theoretical prediction of reactivity describing the electronic structure of the substrate only is delineating that structure in a random environment and its response to random perturbations of the environment. An article of faith among biochemists and biologists, however, is that metabolic processes are not random. Enzymes that are critical for the correct functioning of a biological system are highly specific for their correct substrates. A general assumption, however, is that the microsomal mixed function oxidases are nonspecific, both with respect to variety of substrate and to site of attack. MO calculations (and model in vitro systems, when available) should enable us to test this assumption of the random nature of the microsomal oxidases. Deviations from the predicted reactivity of a substrate toward such oxidation indicate a biological order imposed on the normal chemistry of the compound involved. Consider two carcinogens of considerable significance in current research, 2-acetamidofluorene (AAF) and benzo[*a*]pyrene (BP). Frontier orbital coefficients for AAF predict that the carbon atoms in AAF which should be most susceptible to electrophilic substitution are (in order) 7, 5, 4, and 3. Yet 4-hydroxy(OH)-AAF is not found in the urine of rats treated with AAF (*8*) or in the mixture of products resulting from incubation of AAF with rat liver microsomes in vitro (*9*). A similar order is predicted for free radical attack. For BP, the most reactive position toward electrophilic, radical, or nucleophilic substitution is the 6-carbon, a prediction confirmed by extensive laboratory experience. Yet the predominant metabolite of BP in microsomal incubations is 3-OH-BP (*10, 11*). Even the sum of all metabolites involving oxidation at the 6-position may be less than the amount of either 3-OH-BP or the 9,10-diol (*10, 11*). Unfortunately, even current analytical methods cannot yet resolve completely all possible metabolites of BP. Using the same principles, we can make similar predictions about numerous other compounds; the results should prod investigators into a critical evaluation of metabolic mechanisms. The ability of even the simplest MO techniques to predict relative reactivities in this manner was demonstrated by Dewar and Warford (*12*), who calculated the relative reactivities of positions in phenanthrene toward electrophilic substitution with a paper-and-pencil method. Their predictions were confirmed in a detailed and tedious experiment of nitrating phenanthrene, separating the products, and comparing them with the various nitrophenanthrenes prepared by unambiguous routes. Presumably, the development and use of higher-order methods should permit accurate predictions for many compounds not in the theoretically simple class of polycyclic aromatic hydrocarbons.

Despite the current acceptance of the proposition that nonreactive carcinogens must be metabolized to active forms, the oldest assumption is that unmetabolized carcinogen, particularly polycyclic hydrocarbon, reacts directly with the cellular target controlling neoplasia. This assumption demands that carcinogenic activity be directly correlated with a particular type of reactivity of the carcinogen. Fundamentally, a polycyclic hydrocarbon may undergo two types of reaction: addition or substitution. Either the original complement of carbon and hydrogen atoms is added to, or a substitution is made for one of the hydrogen atoms. A substitution reaction involves only one carbon atom of the hydrocarbon, whereas an addition must involve two carbon atoms. Generally, theorists of chemical carcinogenesis have not recognized that the same intermediate stage of an addition to one carbon atom to produce an unstable species is probably necessary for both types of metabolic events (*13, 14*). Since this is not necessarily the case for laboratory reactions, some confusion and ill will have been created in this area. The Pullmans (*15*) originally considered the possibility that a substitution reaction might be the critical one. However, this hypothesis provided no correlation with carcinogenicity and was temporarily abandoned. The assumption of addition to the most reactive double bond in the hydrocarbon (the "K-region") proved more rewarding, especially when modified by the further assumption that a deactivating addition of the Diels-Alder type could take place (at the "L-region"). The final modification satisfying the Pullmans was the assumption that each addition *has as its initial step a substitution*. The Pullmans (*15*) then combined the predictive indices for the substitution and the addition to obtain complex indices yielding a reasonable correlation with carcinogenicity. The basic assumption of addition as a necessary reaction was not directly challenged for 14 years, at which time two separate papers appeared (*14, 16*). Hoffman (*16*) applied semiempirical calculations to the compounds discussed by the Pullmans and concluded that there was no theoretical basis for choosing addition as the critical reaction. Scribner (*14*) showed that, once the assumption of the importance of a K-region was made, prediction of substitution at the K-region as an activating reaction plus substitution at the L-region as a deactivating reaction proved sufficient to give a correlation as good as that found by the Pullmans, and possible better. Recently, Herndon (*4*) revived the original addition hypothesis, claiming that the use of a novel resonance theory method resulted in a better correlation between predicted and observed levels of carcinogenic activity. He was apparently unaware of Hoffman's work, which rested on calculations of the same degree of accuracy as those of his own. In addition, Herndon's presentation suffers from two critical difficulties. First, he based his correlation on the Iball index. This crude attempt at quantitating carcinogenesis suffers in itself from two defects: 1) it is not based on dose–response studies but rather on the tumor production from an arbitrary uniform dose for all carcinogens; and 2) the relative order of carcinogenicities of compounds often varies with the route of administration and the target organ. The second failing in Herndon's paper is more critical: When based on the simple question of predicting whether a compound will be carcinogenic, his correlation is less reliable than that obtained by Scribner. Thus Herndon's claim that his "results support the idea that the hydrocarbon itself is the ultimate carcinogen" is difficult to accept.

Fukui and co-workers (*17*) recognized one fact that had not impressed the Pullmans: Carcinogenic alkylating agents reacted with nucleophilic centers in the cell. This observation suggested to them that hydrocarbons and aromatic amines also ought to react with nucleophilic centers. This group, however, also proceeded from the assumption that unmetabolized carcinogen reacted directly with the critical site. It is perhaps appropriate to use this example to comment on another attitude that has plagued theorists of chemical carcinogenesis, i.e., a tendency to disregard the realities of chemistry, both metabolic and laboratory. Nucleophilic substitutions on unsubstituted or alkylated polycyclic aromatic hydrocarbons are thermodynamically unfavorable. Under the most extreme laboratory conditions designed to produce such a substitution, it is more likely that an elimination would occur, leading to an unstable benzyne-type intermediate. Even such reactions as these can normally be performed only on hydrocarbons already bearing an appropriate leaving group. A nucleophilic substitution should proceed with even more difficulty (if that is possible) in the ring of an aromatic amine, since the electrons donated by the nitrogen must decrease reactivity toward an electron-bearing reagent (nucleophile). Since such reactions do not occur in vitro under the most vigorous conditions, it is illogical to imagine that they may take place under the mild ones of a biological system. Of course, enzymes do facilitate reactions difficult for a chemist, but even enzymes cannot violate thermodynamics. Nor are enzymes allowed under the original assumption.

REACTIONS OF THE ULTIMATE METABOLITE

If one assumes that, indeed, metabolic activation is necessary for a carcinogen to produce its effect, the rate of production of a specific metabolite might control the level of carcinogenicity. This assumption was the basis of Scribner's approach to hydrocarbon carcinogenicity (*14*), and not that of direct reaction between carcinogen and critical target. Dipple et al. (*13*), however, were the first to attempt to deal theoretically with the known facts concerning metabolism and reactivity of carcinogens. They assumed that the first reaction of a hydrocarbon was an electrophilic oxidation to an intermediate oxygen-bearing sigma complex with a positive charge delocalized throughout the aromatic system. They then postulated that this species could continue to a relatively stable epoxide, or that it could react directly with a critical target and initiate carcinogenesis. The stability of this intermediate would then determine its carcinogenic activity. Unfortunately, this postulate did not result in any useful correlation of theory with carcinogenicity for unsubstituted hydrocarbons. It did produce a remarkably good correlation for methylated benz[*a*]anthracenes and dibenz[*a,h*]-anthracenes, based on the assumption that a carbonium ion is generated at the methyl group. This study is a good example of a valid combination of theoretical approach and experimental testing (which followed). Much fruitful research has followed this particular study, with a new class of carcinogen, the bromomethylbenz[*a*]anthracenes (*18–22*). Unfortunately, the original postulate that a methylated hydrocarbon is activated by generating a positive charge on the methyl group seems to have been refuted by recent work on the binding of hydrocarbons to DNA (*23, 24*). On the other hand, it suggests that, within a closely related series of carcinogens all having the same type of reactivity and similar physical properties, the ability to undergo a particular type of reaction controls the tumor-initiating potency of the carcinogen. The entire program has been an impressive example of a continuing dialogue between fact and theory, leading to significant conclusions concerning the nature of carcinogen chemistry.

Application of MO theory to aromatic amine carcinogenesis is in the fortunate position of having available accurate models for the ultimate reactive metabolites of aromatic amines and/or their *N*-acetyl derivatives. Esters of the *N*-hydroxy derivatives of AAF and 4-acetamidobiphenyl (AABP) have been found to yield the same adducts with DNA and RNA in vitro as those isolated from livers of rats fed *N*-OH-AAF or *N*-OH-AABP (*25, 26*). Kinetics experiments and product studies indicated that the reactive intermediate in these reactions is an *N*-aryl-*N*-acetylnitrenium ion (*27*). MO calculations were successfully used to explain the relative decomposition rates of esters of a series of different *N*-arylacetohydroxamic acids (*27*). Reaction rates did not explain the yield or substrate selectivity from these reactions, however. Recently, we have been able to show that an MO description of the charge distribution in the nitrenium ion may accomplish the latter goal (*28*). Further experiments are required to substantiate this possibility.

CONCLUSIONS

So far no technique for prediction of chemical reactivity from MO (or resonance theory) methods has explained successfully the relative carcinogenicity of an extended series of compounds, except for the bromomethylbenz[*a*]anthracenes. This fact in itself should make us acutely aware of the possibility that the factors governing carcinogenesis may include chemical reaction in only a minor, albeit necessary, role. On the other hand, because so few studies have been undertaken to understand transport, metabolism, and ultimate reactivity by theoretical methods, it is safe to say that this area has not really been explored. For all the benefits collaboration may offer, intimate contact is essential between investigators familiar with MO theory and those familiar with carcinogen chemistry and metabolism. Failure to maintain such contact could easily result in perpetuation of a situation in which theoreticians are not really aware of the complexities of the systems they are being asked to describe, and one in which experimentalists may be unaware of significant studies which would be suggested by theoretical results.

The ultimate ideal in the goals of carcinogenesis research is the ability to predict carcinogenicity from knowledge of chemical structure alone. Yet no progress can be expected in this direction until there is a substantial improvement in the level of research into chemistry of carcinogens and the micromechanisms of metabolism. As I mentioned in the beginning, MO theory is a language. In terms of resources, it will require but a small expenditure to use this language, compared with the resources required to generate the data to be comprehended. In the evaluation of current and projected studies by investigators who wish to use MO theory, we must ask: Does this use of theory suggest specific experiments to be performed or specific classes of data to be collected, which will improve our understanding of carcinogen transport, metabolism, or reactivity? If so, then it appears wise to encourage such use and to improve its accessibility to the carcinogenesis research community.

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ADDENDUM

Since the publication of the original Editorial, significant advances have been achieved in the use of MO theory to aid in understanding carcinogenesis. These have included calculations at the extreme ends of complexity, and they show the power of explanation available with even the Hückel method. Four papers in particular may be cited plus an illustration of the occasional disagreement between intuition and MO results. One of the more difficult problems in understanding the chemistry of carcinogens is explaining the various positions of attack of different carcinogens, both on the target bases and on aromatic carcinogens. Pullman and Armbruster (*1*) made a major advance in this area by using STO-3G calculations to explain the differences of reactivity of the proton and methyl and ethyl cations with cytosine. Using polyelectronic perturbation theory based on Hückel MO's, Scribner and Fisk (*2*) reproduced the mode of reaction of various aromatic carcinogens with guanosine that involved variations in site of reactivity on guanosine and the electrophiles.

With the establishment of diol epoxides as ultimate carcinogenic forms of polycyclic aromatic hydrocarbons (*3*), some investigators have tried to explain the differences in carcinogenicity of hydrocarbons based on this concept. Probably the most useful of these were papers by groups from Wright State University and the University of Chicago. Smith et al. (*4*) calculated reactivity indices for several of the steps in the activation and reaction of hydrocarbons and concluded that initial oxidation is not a distinguishing reaction between carcinogens and noncarcinogens, whereas good correlations were obtained between carcinogenicity and either secondary oxidation or reaction of the final diol epoxide. Since metabolism to epoxides can be followed by rearrangement to phenol, hydration to dihydrodiol, or reaction with macromolecules, it follows that distribution among these various pathways may control carcinogenicity. Fu et al. (*5*) were concerned to show that such distribution could be predicted by MO methods. Using the simple perturbational method of Dewar (*6*), a paper-and-pencil procedure, they obtained good correlations between predicted and observed structures of products formed during isomerization of arene oxides to phenols, hydration and nucleophilic addition to arene oxides, and dehydration of arene dihydrodiols.

A recent metabolic study showed that the major metabolite of 3-acetamidofluorene is 9-hydroxy-3-acetamidofluorene (7). Since such a metabolite is barely detectable from 2-acetamidofluorene, the authors were concerned to achieve an explanation for the difference and believed they found it in the *para* orientation of the amide group to C-9, ostensibly making formal hydride abstraction easier from 3- than from 2-acetamidofluorene. In their explanation, they assert that a canonical resonance form involving C-9 and the amide in the resulting carbonium ion cannot be written for 2-acetamidofluorene. This is incorrect. In addition, Hückel MO calculations for the change in pi-energy in going from the corresponding byphenylamides to the carbonium ions bearing the bridging 9-carbon show that, in fact, 2-acetamidofluorene should be more easily oxidizable at C-9 than 3-acetamidofluorene. If electron distribution does indeed control the differences in metabolism between the two amides, a more likely explanation is that C-7 of 2-acetamidofluorene is a better substrate than C-7 of 3-acetamidofluorene, based on a calculation of superdelocalizability [*see* (*17*) of original Editorial for method].

The National Cancer Institute is now funding MO studies of carcinogens and carcinogen reactions under the contract mechanism, so that it appears likely that the next 5 years will tell us whether the promise of theoretical chemistry in helping us to understand chemical carcinogenesis will be fulfilled. An interesting attitude in this respect surfaced in the paper by Smith et al. (*4*), who viewed this original Editorial as a warning of the pitfalls that could be encountered in trying to explain carcinogenesis with MO theory. Although several questionable approaches were indeed judged deficient, the thrust of the Editorial was intended to be one of encouragement and to prod investigators to enter the forest of carcinogen chemistry with theoretical tools and to attempt to understand the actual chemistry instead of seeking correlations based on no knowledge of in vivo reactions.

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GUEST EDITORIAL .... by K. Gerhard Brand [1]

JNCI 57:973–976, 1976

Diversity and Complexity of Carcinogenic Processes: Conceptual Inferences From Foreign-Body Tumorigenesis [2]

To gain an insight into the general phenomenon of cancer, my colleagues and I have studied FB tumorigenesis (*1*) by analyzing and comparing this model with other forms of carcinogenesis.

BASIC EXPERIMENTS AND RESULTS

Earlier investigators established that the physical presence of the FB material, not its chemical reactivity, causes tumor development and that peculiarities of the FB reaction, especially the degree of fibrosis, strongly influence tumor incidence.

We used the following basic method (*2*) for analyzing the tumorigenic process. Plastic films were implanted into CBA/H or CBA/H-T6 mice. After various times, the implants were excised. Segments of the implants were transferred separately to mice that were histocompatible with the donors, yet distinguishable on the basis of the T6 chromosomes. Sarcomas of donor origin developed in the recipients months or years later, which indicated that preneoplastic cells were on the FB surface at the time of transfer. Tumors that arose from segments of the same original implant were often closely related (homologous) with regard to *a*) latency, *b*) chromosome aberrations, *c*) histopathology and anaplasticity, and *d*) growth characteristics in vivo and in vitro.

These results permitted the following conclusions: Tumors arose from cells with the same neoplastic specificity, which indicated their clonal nature. Specific tumor properties must have been predetermined and fixed in these clonal cells before the implants were cut and transferred. The demonstration of such clones implied prior existence of "parent cells" in which the initial determining event must have taken place.

Further studies revealed the parent cells to be pluripotential mesenchymal stem cells of the microvasculature, most likely pericytes or endothelial cells (*3*). The Most Probable Number of parent cells in the tumorigenic FB reaction was found to be small and to depend on the implant size: one for a 7×15-mm and three for a 15×22-mm implant. These values agreed with those obtained by direct counts (*4*).

Curves of tumor incidence data plotted against time were characterized by multiple, distinct peaks consistent with a composite of several subpopulations (to be published).

Kinetic studies were done in CBA mice to determine the appearance time and the location of parent cells or clonal progeny in relation to the implant and the course of the FB reaction. FB tumorigenesis was found to be a multistage developmental process (*5*). *Stage 1* began with the implantation of the FB and was characterized by an early inflammatory FB reaction with cellular infiltration predominantly by macrophage-type cells. By the 12th day, the FB surface was already covered by a macrophage monolayer. Within 4–8 weeks, preneoplastic parent cells could be demonstrated in the loose cellular FB-reactive tissue, but not on the FB surface among the monolayer cells. The main criterion of *stage 2* was the formation of a fibrous capsule around the FB during the second month post implantation. Preneoplastic cells were then present as clones in the capsule and in the loose connective tissue around it, but still not on the FB surface. *Stage 3* began when preneoplastic clonal cells settled on the FB surface. The timing of this event varied: It was recorded in 1 test animal at 11 weeks post implantation, in 30% of the test animals by the fifth month, in 70% by the sixth month, and in nearly 100% by the seventh month. The fibrotic consolidation of the capsule was complete between the fourth and the sixth month. The macrophage-type cells that still predominated on the FB surface appeared ultrastructurally inactive and dormant. However, FB contact was not at that time a sine qua non for preneoplastic cell maturation. *Stage 4* began and stage 3 ended, when direct FB contact became a requirement for completion of preneoplastic maturation; homologous preneoplastic cells in the capsule would not give rise to tumors upon transfer of the capsule unless a new FB piece was inserted.

PRIMARY ACQUISITION OF NEOPLASTIC DESTINATION AND TUMOR DETERMINANTS

The initial acquisition of neoplastic destination by a parent cell simultaneously creates determinants of specific tumor properties including latency, chromosome aberration, histopathology, degree of anaplasticity, and

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ABBREVIATION USED: FB=foreign body.

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invasiveness. This primary "transforming" key event takes place *a*) before the fourth to eighth week of the FB reaction, *b*) at sites distant from the FB, and *c*) in a single or a few mesenchymal pluripotential stem cells of the microvasculature.

While the key event occurs, parent cells are not found in contact with the FB surface. Thus the FB cannot be incriminated as a direct "transforming" agent. Rather, the key event appears to be intrinsic to the cell. In this regard, FB tumorigenesis seems to differ fundamentally from other forms of carcinogenesis where neoplastic transformation results from direct biomolecular interference or damage by exogenous forces such as chemicals, radiation, or viruses.

The frequency of key events in connection with the FB reaction is low but predictable, which suggests a spontaneous chance event that obeys statistical rules of probability. The sizes of the FB and the FB reaction are positively correlated with the Most Probable Number of parent cells, which stands for frequency of spontaneous carcinogenic key events. This implies that the key event may occur also in cells that proliferate at a low regular renewal or maintenance rate, i.e., in cells of normal unstimulated tissue. Conceivably, parent cells with complete neoplastic determination may be present in tissues even before FB implantation.

The central question concerning the molecular nature of "transforming" carcinogenic key events is a matter of speculation. In FB tumorigenesis, the key event must be variable and complex in that it produces varied combinations of multiple determinants and thus the individuality of heterologous FB-induced tumors. Conversely, any tumor characteristic rooted in one of the primary determinants, e.g., tumor latency, can be assumed to reflect the key event itself. Noncumulative curves of FB-induced tumor incidence show distinct peaks. The overall type of curve does not conform with a normal distribution; rather, it reflects a composite of several subpopulations, each with its own normal distribution curve. The same phenomenon was described and mathematically confirmed by computer analysis also for certain chemical carcinogenesis models (*6, 7*). This may be interpreted to indicate that FB-induced tumors fall into several distinct categories according to inherent differences in latency. Since latency is one of the fixed primary tumor determinants, the class differences must already exist at the parent cell level. Consequently, the carcinogenic key event must be multiform.

Another significant aspect is the heritability and stability of the specific tumor determinants that pass virtually unchanged from the parent cell through numerous preneoplastic clonal generations during tumor latency. This points to genetic–epigenetic–regulatory cell components as probable sites of carcinogenic key events.

Most researchers generally agree that the neoplastic state reflects a disturbance of cellular growth regulation. Our knowledge in this area of cellular physiology with its ramifications into differentiation and development is frustratingly scanty. At present, we can only surmise that multiple functional units cooperate in cellular growth control, each unit probably highly complex. Obviously, numerous molecular reactions, circuits, and switching mechanisms must be involved with feedback loops to maintain continuous checks and balances. A single error in any of the various sensitive points may disturb growth control. The effect may be immediately lethal or incapacitating and life-shortening for the cell; or with the error being heritable and passed on to subsequent cell generations, the ultimate outcome could be the complete breakdown of cellular growth control, resulting in unrestrained neoplastic proliferation.

This leads to the question: What causes such errors? In radiation and chemical carcinogenesis, we are dealing with "hit-and-run" agents; in viral tumors, we are dealing with "gene invaders." But, in FB tumorigenesis, the carcinogenic error seems to result from a spontaneous intrinsic event, possibly *a*) a genome change caused either by mutation or by activation of indigenous viral genes, *b*) chromosome defects or imbalance, *c*) aberrant epigenetic differentiation, etc.

Viruses or viral genomes were searched for in cells of FB-induced tumors (*3, 8*), but no convincing evidence of a causal relationship was found. Immature type C particles or intracisternal type A particles were detected by electron microscopy in FB-induced tumors of certain mouse strains, depending on experimental manipulations during preneoplasia. However, preliminary biochemical studies have so far failed to suggest that these particles have more than a coincidental role.

Chromosome abnormalities, especially an excessive or unbalanced load, are consistently associated with FB tumorigenesis, although no singular criterion has been identified so far (*2, 9*). Most importantly, the aberrations are already fixed before clone formation, presumably at the time of the carcinogenic key event. In most other types of cancer, chromosome aberrations are considered nonspecific accidents that occur during preneoplastic progression or successive tumor evolution.

Why should pericytes or endothelial cells instead of macrophages and fibroblasts become tumorigenic parent cells when the macrophages and fibroblasts are subjected to the same FB-reactive stimuli and actually respond with much more vigorous proliferation and activity? Pericytes or endothelial cells, unlike the other cell types, may have a unique inherent predisposition for the carcinogenic key event. This explanation appears plausible, especially if a chromosome disorder is suspected as the etiologic basis of FB tumorigenesis. It has been suggested that pluripotentiality of stem cells is being preserved beyond embryogenesis by exempting these cells from chromosome stabilization. The price to be paid for pluripotentiality could then well be an increased susceptibility to spontaneous errors at the chromosomal and subchromosomal levels (*10*). Another explanation is offered by the fact that pluripotential mesenchymal stem cells may have a longer proliferative life-span than do differentiated macrophages and fibroblasts (*1, 11*). Even if the probability of spontaneous error was equal in any cell type, the error would be expressed only as long as the predetermined tumor latency fell within the life-span of the affected cell lineage.

EARLY MATURATION OF PRENEOPLASTIC CELLS, INDEPENDENT OF FB CONTACT

The phenomenon of tumor homology observed in preneoplastic transfer experiments proves that these tumor determinants are stable during proliferative clone expansion and cellular preneoplastic maturation. The cells lack freedom of variation and of mutational selective evolution. They can follow only one developmental track.

Although fixed and stable, the tumor determinants are silent for the duration of the latency period of the tumor. This points to the existence of mechanisms that block immediate expression of those determinants. Since latency is one of the predetermined tumor characteristics, the suppressing mechanisms must be closely linked to the determining key event. Furthermore, in view of considerable clone-specific variability of tumor latencies, it must be assumed that the responsible suppressor mechanisms vary also from clone to clone.

Although the existence of some kind of "intracellular molecular clock" is indicated, little can be surmised at this point about the nature and modes of action of these suppressor mechanisms. A recent observation (*11*), however, narrows the range of hypothetical possibilities: Those tumor determinants responsible for loss of growth control are expressed gradually. With advancing tumor latency, the chances increase that aneuploid preneoplastic cells can be isolated from FB-reactive tissue and cultured as pure clones. This acquisition of the ability to be cultured, indicating a lowered threshold of proliferative response, seems to coincide roughly with the appearance of preneoplastic cells on the FB surface.

These experiments revealed another significant phenomenon. While the cells were in culture, the preneoplastic maturation process seemed to decelerate to the point of arrest, despite vigorous propagation. In contrast, the sister cells of the same clone that were left in the animal continued their preneoplastic maturation, although they proliferated at a much slower pace. This observation allows two conclusions: 1) Progress of cellular preneoplastic maturation does not simply correlate with number of consecutive cell divisions; this speaks against proliferative dilution or extinction of a preneoplastic suppressor or compensator substance. 2) The forces promoting cellular preneoplastic maturation are not present or not operative under in vitro conditions but only in the in vivo situation.

The second conclusion brings up another question: Which in vivo factors are specifically involved in the preneoplastic maturation process? Most conspicuous is the association of preneoplastic advancement with the state of fibrosis that develops in reciprocity with acute cellular inflammation and macrophage activity. During fibrotic conversion, the FB-reactive tissue contracts so that the capillaries are obliterated. Disconnected, normal pericytes and endothelial cells may then cease to proliferate. The aneuploid preneoplastic cells, however, having a lowered threshold of proliferative response continue to divide and disseminate.

Other in vivo factors, such as chronic anoxia or impaired metabolite exchange within the fibrotic FB capsule, are probably of little, if any, importance (*12*).

The suggestion that the firmly encapsulated implant area is an "immunologically privileged site" (*13*) is without basis because FB-induced sarcomas either possess only weak tumor-specific antigens or lack them altogether at cellular sites accessible to the immune defense mechanisms of the body. Moreover, histocompatibility antigens are frequently lost from cells during neoplastic proliferation as well as during the preneoplastic phases (*14*).

LATE MATURATION OF PRENEOPLASTIC CELLS, DEPENDENT ON FB CONTACT

The cellular event that removes the final barrier on the way to neoplastic autonomy seems to be contingent on the cell's contact with the FB surface. A related discovery was reported by Boone (*15*) who found that tumors were produced in mice by 3T3 cells only when the cells were attached to glass beads. I suggest that these cells represent incompletely matured preneoplastic cells like those responsible for FB-induced sarcomas. To reach neoplastic autonomy, such cells still require the final FB-dependent maturation step. This suggestion appears all the more plausible, since the 3T3 cells are now assumed to be descendants of pericytes or endothelial cells (*16*).

The surface effects of the FB on the attached cell may be basically of a general physical nature because differences in size or surface properties of the FB are of little consequence. Preneoplastic cells that had proceeded through the initial event and the contact-independent maturation phases were found to pass through the final stage with equal speed, whether the FB was large or small and whether it was of smooth plastic, roughened plastic, or glass (*17*).

The cell structure primarily involved in this final contact-dependent maturation process is obviously the cell membrane. Systematic investigations are lacking, but various physical or physicochemical forces may be involved. Also, the FB may interfere with intercellular contact or molecular communication, as suggested by studies of tumor induction by Millipore filters (*18*).

One observation has been reported by various authors (*4, 19, 20*) which must be interpreted as an indication that terminal surface dependency of the preneoplastic maturation process is not without exception. When an FB implant is removed from the animal before a tumor has developed, the tumorigenic process is usually aborted, but occasionally tumors develop. There may be several explanations. If the interval between FB removal and tumor appearance was short, the tumorigenic process might have progressed far enough so that surface attachment of the preneoplastic cells had taken place by the time of FB removal. In this situation fully matured early tumor cells, which either had already detached from the FB or were mechanically stripped off during the removal procedure, stayed behind in the capsule. In other instances the fibrotic capsule may not have resolved after FB removal but may have persisted as a firm chronic scar. Frequently plaques of mineral deposits or osseous metaplasia (*20*) were detected in the scar tissue which could have exerted a surface effect on maturing preneoplastic cells similar to that of an FB.

GENERAL CONSEQUENCES AND INVESTIGATIONAL PROSPECTS

Two fundamental assumptions derived from the studies on FB tumorigenesis may have significance in cancer research.

a) The primary carcinogenic event does not seem to occur uniformly at one specific locus but may do so at various sensitive points within the cellular growth control system. Diversity of carcinogenic key events should be least expected in FB tumorigenesis, since the agent introduced lacks cell invasiveness and biologic activity and actually does not even physically interact with target cells at the time of the key event. All the more it seems plausible that chemical and viral carcinogens, radiation, and similar cell-invasive agents would affect still other target points.

b) Ultimate expression of specific tumor characteristics, as predetermined by the initiating key event, and arrival at the stage of neoplastic autonomy require the cells to pass through preneoplastic maturation phases. This intracellular developmental process depends on a stringent sequence of extracellular environmental conditions reminiscent of induction phenomena described for normal cell and tissue differentiation. Some factors involved are effective only in vivo and have not been simulated in a simple tissue culture environment.

In general, several theoretical and practical consequences ensue.

1) A defect in the cellular growth control system appears central to every carcinogenic process. This implies that a comprehensive understanding of carcinogenic processes may remain out of reach as long as the mechanisms of normal cell regulation and growth control have not been elucidated.

2) Numerous groups of agents with diverse modes of action are known to cause cancer. The initial affection of the cell and also the cellular sites at which the primary events take place must be as varied. Yet the end result, cellular neoplastic autonomy, is obviously the same. Hence some segment of the developmental cell path toward neoplastic autonomy must be common to all forms of carcinogenesis. The extent of such carcinogenic uniformity is a matter of basic controversy. Certain virologic hypotheses imply that, by activation of a specific inherent oncogenic virus or genome, the carcinogenic process is a universal one at the primary event and thereafter. In contrast, the observations on FB tumorigenesis in context with findings on other tumor induction models suggest that the only common event may be the terminal breakdown of the cellular growth control system. The collapse of this multifunctional composite could come about in many different ways which may be targeted at one of various interlocking functional subunits after having been initiated by one of many possible key events.

3) The model of FB tumorigenesis appears particularly suited for studying the multifactorial in vivo process of carcinogenesis. While the natural preneoplastic events and developments run their course in the animal, cell samples can be periodically taken for culture, cloning, and expansion (*11*). Analytic results obtained on such cell preparations at different preneoplastic maturation stages can be related retrospectively, in the case of homology, to specific tumor characteristics.

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ADDENDUM

In this Editorial, reference was made to research data that have now been published.

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GUEST EDITORIAL

Lee W. Wattenberg[1]

JNCI 60:11–18, 1978

Inhibition of Chemical Carcinogenesis

Current data suggest that chemical carcinogens have a significant role in the etiology of cancer in man. In this editorial, information will be presented concerning many compounds that have the capacity to inhibit the neoplastic effects of chemical carcinogens when administered either prior to exposure to the carcinogen or at the same time. Overall, this inhibition entails prevention of the active form of the carcinogen from reaching or reacting with the target site. Inhibition involving reversal of early phases of the carcinogenic process will not be included. This type of inhibition has been achieved with retinoids and has been reviewed by Sporn et al. (*1*). Prevention of neoplasia by blocking of the formation of carcinogens, as has been accomplished for nitroso compounds, likewise will not be dealt with (*2*). Most of the inhibitors to be discussed are synthetic compounds; some are constituents of natural products, including vegetables consumed by man (*3–5*). The inhibitors identified thus far show a great diversity of chemical structures, making it likely that we have only a limited knowledge of the total spectrum of compounds having this property. This diversity increases the probability that inhibitors have or could be made to have an impact on the response of humans to chemical carcinogens.

The following are some potential mechanisms of inhibition of chemical carcinogenesis:

1) Alteration of metabolism of the carcinogen:
    - *a*) Decreased activation
    - *b*) Increased detoxification
    - *c*) Combination of (*a*) and (*b*)
2) Scavenging of active molecular species of carcinogens to prevent their reaching critical target sites in the cell
3) Competitive inhibition.

However, much of the information that has actually been obtained on inhibitors has come from empirical studies. Some inhibitors were originally identified as a result of work based on postulated mechanisms of inhibition. Even in these instances, as will become evident, there is frequently doubt about how correct the postulated mechanism really is. Thus the amount of phenomenologic data is considerable, but solid information on mechanisms of inhibition is scarce. As a result, the organization of the following presentation is somewhat arbitrary and is formed around groups of inhibitors having certain common features. After discussion of 8 such groups, some general considerations of problems and potentials of compounds that inhibit chemical carcinogenesis will be studied.

INHIBITORS OF CHEMICAL CARCINOGENESIS

Phenolic Antioxidants and Ethoxyquin

The use of antioxidants as possible inhibitors of the chemical carcinogens has been based in general on the concept that the antioxidants may exert a scavenging effect on the reactive species of carcinogens, thus protecting cell constituents from attack. In early studies, wheat germ oil and α-tocopherol were employed. Experiments showing positive and negative results have been published. Confirmatory reports on the positive experiments have not appeared, so that the implications of this work are not clear. These investigations as well as our own experience with α-tocopherol, which has not shown it to be inhibitory, have been summarized (*6*). However, under some appropriate conditions, an inhibitory effect possibly does occur.

During the past several years, studies have been performed with other antioxidants. The most extensive work of this type has been done with phenolic compounds, in particular, BHA and BHT. Inhibition occurs under a variety of experimental conditions and with a broad range of chemical carcinogens as shown in table 1. Several nonphenolic antioxidants inhibit chemical carcinogenesis. One of these is ethoxyquin, a widely used antioxidant commonly added to commercial animal feed.

Some studies of mechanism of inhibition by BHA and BHT of chemical carcinogenesis have been per-

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Abbreviations used: BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene; BP = benzo[*a*]pyrene; DMBA = 7,12-dimethylbenz[*a*]anthracene; DENA = diethylnitrosamine; FAA = *N*-2-fluorenylacetamide; N-OH-FAA = *N*-hydroxy-*N*-2-fluorenylacetamide; N-OH-FA = *N*-hydroxy-*N*-2-fluorenylamine; BP-4,5-oxide, BP-9,10-oxide, BP-7,8-oxide = oxides of BP; 3-HOBP = 3-hydroxybenzo[*a*]pyrene; DMH = *sym*-dimethylhydrazine; DMN = dimethylnitrosamine; MCA = 3-methylcholanthrene.

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TABLE 1.—*Inhibition of carcinogen-induced neoplasia by phenols, lactones, and ethoxyquin*

Carcinogen	Inhibitor [a]	Species	Site of neoplasm inhibited
BP	BHA (*7*), ethoxyquin [b]	Mouse	Lung
BP	BHA (*6*), BHT (*6*), coumarin, [b] α-angelicalactone [b]	Mouse	Forestomach
DMBA	BHA (*7*)	Mouse	Lung
DMBA	BHA (*6*), ethoxyquin (*6*)	Mouse	Forestomach
DMBA	BHA (*8*), BHT (*8*)	Mouse	Skin
DMBA	BHA (*6*), BHT (*6*), ethoxyquin (*6*), coumarin (*9*)	Rat	Breast
7-Hydroxymethyl-12-methylbenz[*a*]anthracene	BHA (*7*)	Mouse	Lung
Dibenz[*a,h*]anthracene	BHA (*7*)	Mouse	Lung
DENA	BHA (*10*), ethoxyquin (*10*)	Mouse	Lung
4-Nitroquinoline-*N*-oxide	BHA (*10*), ethoxyquin (*10*)	Mouse	Lung
Uracil mustard	BHA (*7*)	Mouse	Lung
Urethan	BHA (*7*)	Mouse	Lung
FAA	BHT (*11*)	Rat	Liver
N-OH-FAA	BHT (*11*)	Rat	Liver, breast
4-Dimethylaminoazobenzene	BHT (*12*)	Rat	Liver
Azoxymethane	BHT (*13*)	Rat	Large intestine

[a] *Numbers in parentheses*=reference numbers.
[b] Wattenberg LW: Unpublished observations.

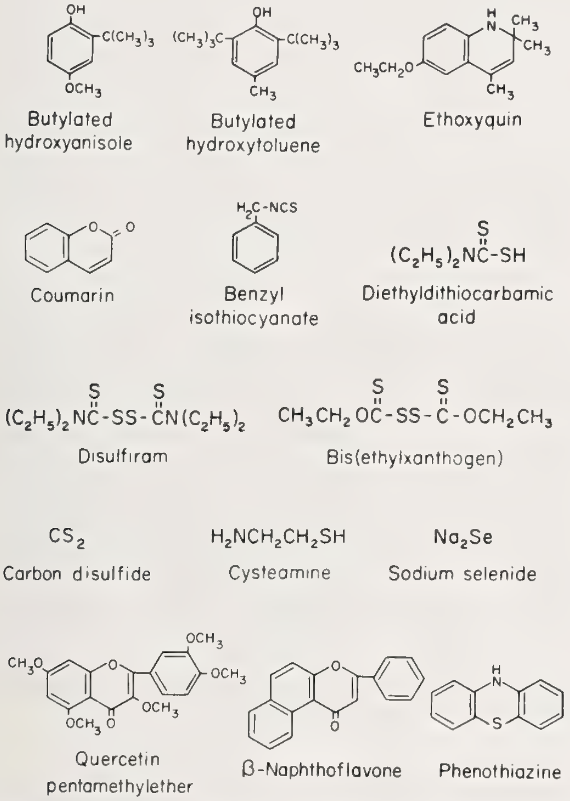
formed. One set has been aimed at the determination of the mechanism of inhibition by BHA of BP-induced neoplasia. BP is metabolized by the microsomal mixed-function oxidase system which acts on a wide variety of xenobiotic compounds, including polycyclic aromatic hydrocarbons. Reactive metabolites as well as detoxification products are produced. The effects of administration of BHA on microsomal metabolism of BP in female A/HeJ mice has been studied with experimental conditions similar to those in which BHA inhibits neoplasia due to this carcinogen. Incubation of BP and DNA with liver microsomes from the BHA-fed mice results in approximately half the binding of BP metabolites to DNA as compared to that found with microsomes from control mice (*14*). Investigations have been undertaken to determine if the BP metabolites that are formed when this carcinogen is incubated with liver microsomes prepared from BHA-fed mice differ from those formed in the controls. Of major interest were the effects of BHA feeding on BP-epoxide formation. Formation of BP-4,5-oxide, which can be measured directly by high-pressure liquid chromatography, was reduced with microsomes from BHA-fed mice. BP-9,10-oxide and BP-7,8-oxide cannot be measured directly because of instability. However, data based on summation of diols and phenols resulting, respectively, from the enzymatic and spontaneous conversions of these oxides indicate that they are present in reduced amounts in the microsomal incubations from BHA-fed mice. The major metabolite in microsomal incubations from BHA-fed and control mice was 3-HOBP. This metabolite constituted a significantly higher percentage of the total metabolites formed when BP was incubated with microsomes from BHA-fed mice, as compared to the percentage in control mice. Thus BHA administration causes two metabolic alterations that could result in an inhibitory effect on BP-induced carcinogenesis: 1) a decrease in epoxidation, which is an activation process, and 2) an increase in 3-HOBP, a metabolite of detoxification (*15*).

Studies of the mechanism of inhibition of polycyclic hydrocarbon-induced skin carcinogenesis by phenolic antioxidants have been performed by Slaga and Bracken (*8*). In this work, BHA or BHT was applied topically. The animals were killed 3 or 12 hours later, and the binding of [^{3}H]BP or [^{3}H]DMBA to DNA was determined in epidermal homogenates. Both carcinogens showed approximately half as much binding to DNA in homogenates from mice that had received BHA or BHT 3 hours before death than in homogenates from control mice. The inhibition of binding was still apparent at 12 hours but was a lesser order of magnitude.

An early investigation aimed at determining the mechanism of inhibition of carcinogenesis of FAA and N-OH-FAA by BHT was done by Grantham et al. (*16*) who found that administration of BHT in the diet increased excretion of each carcinogen in the urine. This higher level of excretion was accounted for chiefly by glucuronic acid conjugates. Animals receiving BHT showed lower levels of radioactivity in blood, liver, and liver DNA 48 hours after injection with labeled carcinogen. Thus BHT appears to increase detoxification of FAA and N-OH-FAA by enhancing conjugation and thereby reducing the pool of metabolites available for activation reactions.

At the present time BHA probably is the most versatile and least toxic inhibitor of chemical carcinogenesis that has been identified. Although BHT also inhibits a variety of carcinogens, unfortunately it has some noxious properties, including tumor promotion (*17*). An important difference between the two compounds is the nature of the substitution para to the hydroxyl group (text-fig. 1). In BHT this position has a methyl group that oxidizes to toxic metabolites. In BHA, a methoxy group is present in the para position. If metabolism occurs, it is a hydrolysis. The resultant phenolic group can be conjugated, which facilitates the excretion of the compound.

BHA is of interest because of its extensive use as an additive in food for human consumption. Studies of mice have been done in which BHA was added to the diet with BP, a carcinogen widely encountered in the environment. At a concentration of 5 mg BHA/g diet, the carcinogenic effect of 1 mg BP/g diet on the forestomach of the mouse is inhibited. In the United States, the human consumption of BHA is of the order of several milligrams a day. If one assumes that the results of animal experiments hold for man, this amount of the antioxidant could be important in the inhibition of the effects of chronic exposure to low doses of carcinogens, the type of exposure that is most likely to occur in human populations.



TEXT-FIGURE 1.—Some compounds inhibiting chemical carcinogenesis.

Disulfiram and Related Compounds

Experimental studies of the capacity of disulfiram (Antabuse; tetraethylthiuram disulfide) and some related compounds to inhibit chemical carcinogenesis have been done with the same experimental models employed for the phenolic antioxidants (table 2). These sulfur-containing compounds are potent inhibitors of BP-induced neoplasia of the forestomach. Work aimed at elucidation of the mechanism of disulfiram inhibition has shown that administration of disulfiram in the diet reduces binding of [^{3}H]BP and [^{14}C]BP to DNA, RNA, and protein of the forestomach (*18*). Disulfiram and related compounds are interesting in their effects on carcinogen-induced neoplasia of the large intestine (*3, 19, 20*). When added to the diet, disulfiram, diethyldithiocarbamate, and bisethylxanthogen profoundly inhibit large bowel neoplasia resulting from sc administration of DMH. Work has been done with azoxymethane, an oxidative metabolite of DMH, which also produces neoplasia of the large intestine. Under experimental conditions comparable to those used with DMH, disulfiram has also been found to inhibit azoxymethane-induced neoplasia of the large intestine but to a considerably lesser extent than with DMH as the carcinogen (*19*).

Studies bearing on the mechanism of inhibition of neoplasia of the large bowel by DMH and azoxymethane have shown that disulfiram inhibits the oxidation of both of these carcinogens in vivo (*27–29*). Work concerning the question of whether the inhibitory function resides in the intact molecule of disulfiram or a metabolite of this compound has been done (*28, 29*). These investigations have demonstrated that CS_2, a metabolite of disulfiram, inhibits the oxidation of DMH and azoxymethane. The data obtained suggest that CS_2 may be the chemical species responsible for the inhibitory action of disulfiram and related compounds. Others have reported that incubation of microsomes with CS_2 in the presence of NADPH results in covalent binding of the sulfur to the microsomes, with an accompanying decrease in cytochrome P_{450} as measured spectroscopically

TABLE 2.—*Inhibition of carcinogen-induced neoplasia by sulfur-containing compounds*

Carcinogen	Inhibitor [a]	Species	Site of neoplasm inhibited
BP	Disulfiram (*18, 21*), bisethylxanthogen (*19*), 2-chloroallyl diethyldithiocarbamate (*19*), *S*-propyl dipropylthiocarbamate (*19*)	Mouse	Forestomach
BP	Benzyl isothiocyanate (*22*), phenethyl isothiocyanate (*22*)	Mouse	Forestomach
DMBA	Disulfiram (*19*), benzyl isothiocyanate (*22*), phenethyl isothiocyanate (*22*), phenyl isothiocyanate (*22*), benzyl thiocyanate (*22*), cysteamine (*23*)	Rat	Breast
1,2-Dimethylhydrazine	Disulfiram (*3, 19, 20*), diethyldithiocarbamate (*3*), bisethylxanthogen (*19*), carbon disulfide [b]	Mouse	Large intestine
Azoxymethane	Disulfiram (*19*)	Mouse	Large intestine
FAA	1-Naphthylisothiocyanate (*24*)	Rat	Liver, ear duct
Ethionine	1-Naphthylisothiocyanate (*24*)	Rat	Liver
3′-Methyl-4-dimethylaminoazobenzene	1-Naphthylisothiocyanate (*25*)	Rat	Liver
4-Dimethylaminoazobenzene	2-Naphthylisothiocyanate (*26*)	Rat	Liver

[a] *Numbers in parentheses*=reference numbers.

[b] Wattenberg LW, Fiala ES: Unpublished observations.

(*30, 31*). When incubated with microsomes under comparable conditions (*31*), several thiono-sulfur-containing compounds, including disulfiram and diethyldithiocarbamate, produce a similar decrease in cytochrome P_{450}. This finding raises the possibility that thiono-sulfur-containing compounds as a group may have the potential to modify cytochrome P_{450} so as to alter the microsomal metabolism of DMH, azoxymethane, and possibly other carcinogens in a manner that decreases their carcinogenicity.

The carcinogen-inhibiting effects induced by disulfiram and diethyldithiocarbamate have drawn attention to the possibility that a number of widely used pesticides having dithiocarbamate or thiocarbamate groups might have similar properties; several have been tested. Two of these, i.e., *S*-propyl dipropylthiocarbamate (Vernolate) and 2-chloroallyl diethyldithiocarbamate, when added to the diet, inhibited BP-induced neoplasia of the forestomach of the mouse. An additional pesticide, bisethylxanthogen (Bexide) also exerted an inhibitory effect in this test system. Thus far, bisethylxanthogen is the only one of these pesticides studied for its effects on DMH-induced neoplasia of the large bowel. Its inhibitory potency is similar to that of disulfiram (*19*).

Bisethylxanthogen has a feature that makes it of particular interest: The molecule does not contain nitrogen (text-fig. 1). Structurally similar dithiocarbamate pesticides have been shown to form nitrosamines, representing a hazard not occurring with bisethylxanthogen. A second relationship between disulfiram and nitrosamines has been reported recently (*32*): Disulfiram influences the organotrophy of DENA and DMN. In the case of DENA, disulfiram added to the diet inhibits liver tumor formation but enhances neoplasia of the esophagus. With DMN, neoplasia of the liver is again suppressed, but tumors of the paranasal sinuses are increased.

Organic Isothiocyanates and Organic Thiocyanates

With studies of the inhibitory capacities of sulfur-containing antioxidants, experiments with benzyl isothiocyanate, phenethyl isothiocyanate, and benzyl thiocyanate have been performed (*22*). These three compounds are naturally occurring constituents of edible cruciferous plants (*33, 34*). Phenyl isothiocyanate, a fourth compound included in some experimental work, is synthetic. The inhibitory effects of these compounds are presented in table 2. 1-Naphthylisothiocyanate and 2-naphthylisothiocyanate have both been shown to suppress the neoplastic effects of azo dyes on the liver. Of interest are studies showing that 1-napthylisothiocyanate alters cytochrome P_{450} in a manner comparable to that of thiono-sulfur-containing compounds (*30*).

Lactones

Coumarins are constituents of a wide variety of plants, including vegetables, used for human consumption (*35*). The administration of coumarin by oral intubation inhibits DMBA-induced mammary tumor formation (*9*). Additional studies have been done with BP-induced neoplasia of the forestomach as the test system. When added to the diet, coumarin, 6-nitrocoumarin, and α-angelicalactone inhibited forestomach tumor formation (Wattenberg LW: Unpublished observations).

Selenium Salts

Inhibition of chemical carcinogenesis by selenium salts has been reported. In an initial paper, the experimental system employed consisted of initiation of epidermal neoplasia with DMBA followed by promotion with croton oil; sodium selenide added to the croton oil suppressed the development of skin tumors (*36*). In subsequent work, MCA was repeatedly applied to the skin. Again, addition of sodium selenide inhibited epidermal neoplasia. In a further experiment, mice were placed on a selenium-deficient diet (Torula yeast) without supplements or with added sodium selenide or sodium selenite. BP was applied to the skin daily to produce epidermal neoplasia. A slight inhibition was found with both of the selenium salts (*37*).

Recently, the addition of sodium selenite to the drinking water has been shown to inhibit large bowel neoplasia in the rat, which results from administration of DMH or methylazoxymethanol acetate (*38, 39*). In additional works, selenium was found to reduce mutagenicity of FAA, N-OH-FAA, and *N*-hydroxy-*N*-2-fluorenylamine for the *Salmonella typhimurium* T-1538 histidine mutant (*39*). Evidence has been presented showing an inverse relationship between *a*) the amount of selenium in soil and forage crops and *b*) human cancer death rates in the United States and Canada in 1965. Likewise, an inverse relationship between human blood levels of selenium and human cancer death rates in several cities has been reported (*40*).

Inducers of Microsomal Mixed-Function Oxidase Activity

Several studies have demonstrated that protection against chemical carcinogens by the administration of inducers of increased microsomal mixed-function oxidase activity is possible (table 3). The inducers employed have varied from compounds such as polycyclic hydrocarbons, which are noxious agents, to chemicals such as flavones, which have little toxicity (*41, 42*). In early studies (*43, 44*), administration of polycyclic hydrocarbon inducers inhibited the occurrence of hepatic cancer resulting from feeding 3′-methyl-4-dimethylaminoazobenzene. Likewise, polycyclic hydrocarbon inducers can markedly reduce the incidence of tumors of the liver, mammary gland, ear duct, and small intestine in rats fed FAA or 7-fluoro-*N*-2-fluorenylacetamide (*44*).

More recently, studies have been done in which protection against the carcinogenic effects of a number of other carcinogens has been observed (table 3). Considerable work has been done with two polycyclic hydrocarbon carcinogens, DMBA and BP. With the use of the pulmonary adenoma test system in the mouse, flavone inducers have been shown to inhibit lung tumor formation resulting from oral administration of DMBA or BP (*41, 42*). An experimental model that has been

TABLE 3.—*Inhibition of carcinogenesis by inducers of increased microsomal enzyme activity*

Carcinogen	Inducer [a]	Species	Organ
3′-Methyl-4-dimethylaminoazobenzene	Polycyclic hydrocarbons (*43, 44*), α-benzene hexachloride (*45*), polychlorinated biphenyls (*46*)	Rat	Liver
FAA	Polycyclic hydrocarbons (*44*), polychlorinated biphenyls (*45*)	Rat	Liver, breast, small intestine
4-Dimethylaminostilbene	Polycyclic hydrocarbons (*47*)	Rat	Ear duct
Urethan	Phenobarbital (*48–50*), chlordane (*50*)	Mouse	Lung
BP	β-Naphthoflavone (*42*), quercetin pentamethylether (*42*)	Mouse	Lung
BP	β-Naphthoflavone (*42*)	Mouse	Skin
DMBA	β-Naphthoflavone (*41*)	Mouse	Lung
DMBA	β-Naphthoflavone (*41*), phenothiazines (*51*), polycyclic hydrocarbons (*52, 53*)	Rat	Breast
Aflatoxin	Phenobarbital (*54*)	Rat	Liver
Bracken fern	Phenothiazine (*55*)	Rat	Small intestine, bladder

[a] *Numbers in parentheses*=reference numbers.

widely used in studies of the effects of inducers of increased mixed-function oxidase activity has been mammary tumor formation in rats given DMBA. Several different types of inducers administered prior to DMBA will inhibit tumor formation. These include: polycyclic hydrocarbons (*52, 53*), phenothiazines (*51*), and flavones (*41*).

The experiments listed in table 3 show a protective effect from administration of inducers of increased mixed-function oxidase activity; however, an apparently conflicting set of data should also be discussed. For many chemical carcinogens, the microsomal mixed-function oxidase system has been demonstrated to convert these compounds to a proximate carcinogenic form (*56*). An initial thought might be that if a compound is activated by an enzyme system to a noxious form, then enhancement of the activity of this system would result in greater damage to the organism. This phenomenon is true in situations involving a reversible effect in which a substantial threshold exists. Rapid activation could be important in achieving such a threshold. However, for chemical carcinogenesis, different conditions exist. In this instance, there appears to be either no threshold or a very low threshold (*57*). Thus one might anticipate that slow activation would result in an equal or even a greater carcinogenic effect than does rapid activation. With slow activation, wastage of activated species of carcinogen from cells would be less likely to occur from production of an excess amount over that most effective for the number of critical binding sites available at a particular time. In addition, active carcinogenic species would be present over a longer period and, therefore, would be more likely to exist at a critical time or times in the cell cycle.

Another factor that may be important in the explanation of carcinogen inhibition by induction of increased mixed-function oxidase activity is that in many instances this system subjects chemical carcinogens to detoxification reactions as well as to activation. The classic example of this double effect is found with the aromatic amines. With these compounds, ring hydroxylation results in detoxification, whereas hydroxylation of the nitrogen is an activation reaction (*58*). Thus administration of inducers of mixed-function oxidase activity in these instances may cause a relatively greater proportion of the carcinogen to be detoxified rather than activated to a carcinogenic metabolite. Changes in proportion of detoxified metabolites to carcinogenic metabolites could simply be the result of relative responses of the two pathways to the inducer. An alternative possibility suggested by the studies with BHA is that a more basic alteration in metabolism may occur, resulting in a changed metabolite pattern. In this instance, the change in metabolite pattern could be independent of the magnitude of induction and, as in the case of BHA, might occur without any overall increase in mixed-function oxidase activity. Further work is required to ascertain the mechanism of action of the compounds discussed in this section which have already been shown to have the combined properties of increasing mixed-function oxidase activity and inhibiting chemical carcinogenesis.

Phenobarbital induces increased mixed-function oxidase activity, an increase in endoplasmic reticulum, and liver enlargement. When administered prior to the carcinogen, it suppresses neoplasia (table 3). However, if it is given subsequent to the carcinogen, the neoplastic response may be enhanced (*59*). This cocarcinogenic effect represents a hazard that requires evaluation with respect to other inducers.

Inhibitors of Microsomal Mixed-Function Oxidase Activity

If the activity of the microsomal mixed-function oxidase system were totally absent, carcinogens requiring activation by this system would not produce a neoplastic effect. Efforts have been made to inhibit chemical carcinogenesis by this mechanism. Studies of suppression of polycyclic hydrocarbon-induced neoplasia have been done with 7,8-benzoflavone (α-naphthoflavone), a potent inhibitor of microsomal mixed-function oxidase activity. In experiments in which DMBA was the carcinogen, epidermal neoplasia has been inhibited (*60*). A problem with exploitation of inhibition of mixed-function oxidase activity as a means of suppressing chemical carcinogenesis is that it would render the organism more susceptible to the noxious effects of xenobiotic compounds detoxified by this system.

Physiologic Trapping Agents

Active forms of chemical carcinogens bind to a wide variety of physiologic compounds. Their carcinogenic potential resides in such reactivity occurring at selective sites. However, if the carcinogenic agent is trapped by other cell nucleophiles, protection might occur. Numerous biochemical compounds contain nucleophilic groups. These have been discussed by Miller and Miller (*61*). A question arises as to whether the amount of one or more of such nucleophiles might be increased so as to protect against chemical carcinogens. A compound of particular interest in this regard is glutathione, which is an excellent trapping agent. However, the level of glutathione has been difficult to increase by experimental procedures. In contrast, a number of extrinsic factors, particularly administration of toxic compounds, can deplete glutathione. The levels and control mechanisms of protective cell nucleophiles could be of great importance in the response to chemical carcinogens. Work aimed at a fuller understanding and exploitation of this potential defense certainly is warranted.

DISCUSSION

With the knowledge that inhibitors of chemical carcinogenesis exist, two questions arise. The first relates to the current role that these compounds have in reducing the impact of environmental carcinogens on man. The second is the optimal role that they might have. To assess their current role, additional information is required as to the full spectrum of compounds in the environment that have the capacity to inhibit carcinogens. A perusal of text-figure 1 and tables 1–3 shows that compounds with a broad range of chemical structures can inhibit chemical carcinogenesis. The diversity indicates that this capacity to inhibit does not reside in restricted chemical characteristics and suggests that other inhibitors that have not yet been identified almost certainly exist. The quest to identify these inhibitors is important in order to correctly account for their impact. Also of critical importance is a full elucidation of mechanisms of inhibition. Such an understanding could provide information that could assist in the identification of compounds in the environment that are inhibitors. In addition, mechanisms of inhibition that entail measurable biochemical parameters could provide a basis for assessment of the susceptibility of particular population groups or individuals to neoplasia from chemical carcinogens.

An important aspect of the evaluation of the role of inhibitors is a consideration of toxicity. Inhibitors currently identified have, to a greater or lesser extent, other biologic activities. A number have toxic properties. Some are even carcinogens or cocarcinogens. However, noxious properties clearly can be dissected away for the basic mechanisms of inhibition. Thus among phenolic inhibitors, BHA is considerably less toxic than BHT and is a more effective inhibitor. In early studies in which protection was obtained by induction of increased microsomal mixed-function oxidase activity, the potent carcinogen MCA was used. Flavones and phenothiazines cited in table 3 inhibit by inducing increased mixed-function oxidase activity, but they are not carcinogens. As more is learned about the basic requirements for inhibition, compounds with increasing inhibitory potency and fewer side effects probably can be found. This point should be stressed, since the currently available inhibitors are a first-generation group. Many have been discovered by chance, or if a particular mechanism was being explored, compounds that were readily available were used. More complete information should enable the design of inhibitors in which unnecessary biologic activities are pealed away, providing less toxic and more effective compounds.

Considerations of the optimal role that inhibitors of carcinogenesis might play entail evaluations of their deliberate use. At present, such a meaure would clearly be premature, because we simply do not have an adequate base of information. However, at a future time when more data are available on mechanisms of inhibition, diversity of inhibitors, and their toxicity, this course of action might be entertained. Accordingly, there would be some value in considering possible criteria that would have to be met prior to deliberate usage of inhibitors of chemical carcinogenesis. I believe that the following criteria are prerequisite to seriously considering deliberate exposure of human populations to a particular inhibitor of chemical carcinogenesis:

Populations with “normal” risk of exposure to chemical carcinogens:

1) The inhibitor has no toxicity or trivial toxicity and/or
2) Human population groups have been exposed inadvertently to the inhibitor (a “natural” experiment in man), and it has been shown to be effective in reducing the incidence of carcinogen-induced neoplasia in appropriate epidemiologic studies. In addition, no evidence of the toxic properties are associated with the inhibitor.

Populations with high risk of exposure to chemical carcinogens:

1) Use of an inhibitor for which there is evidence that the benefits outweigh risks.

For any normal group of individuals, a critical restraint is the possibility of toxicity. An inhibitor would have to be taken by individuals for many years to be effective, so that even a low toxicity could outweigh any benefits. However, selected situations occur in which this formidable obstacle might be overcome. One specific instance entails carcinogens within the gastrointestinal tract. In this instance, an inhibitor that would not be absorbed could be designed. Under such conditions, a compound with little or no toxicity might be available. The importance of these types of considerations is made real by recent findings of mutagenic substances in the feces (*62*). If these substances are in fact carcinogens, as well as mutagens, efforts at finding effective inhibitors active within the large bowel might be warranted. In other sites, specific situations amenable to selective approaches might exist as well.

A second basis for introduction of an inhibitor into the environment would be the acquisition of favorable data from epidemiologic investigations. Such data would include firm evidence that a population group with a significant intake of a particular inhibitor has a diminished incidence of one or more neoplasms. Mechanistic data relating the intake of the inhibitor to carcinogen inhibition, i.e., tissues from the particular population group showing an increased capacity to detoxify carcinogens, would be important. In addition, lack of toxicity from the inhibitor should be clearly evident. Under these conditions, consideration of the use of the material bringing about the inhibition would be warranted. This set of circumstances, in essence, forms a natural or unplanned type of experiment. Depending on the magnitude of the inhibition and reliability of estimates of lack of adverse side effects, convincing data could be provided for deliberate use of the substance.

Population groups with elevated exposures to chemical carcinogens do exist. For individuals of this type, less rigid requirements for lack of toxicity of inhibitors might be justified. With regard to this possibility, an exceedingly important prohibition is that inhibitors should not be used as mechanisms for allowing increased exposures to carcinogens or for increasing tolerance levels to cancer-producing substances.

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ADDENDUM

In the original Editorial, the diversity of inhibitors of neoplasia was stressed. An implication of this diversity is that it is highly probable that other inhibitors exist, which is true. In this Addendum, additional inhibitors will be described.

Indoles

A number of cruciferous vegetables including Brussels sprouts, cabbage, cauliflower, and broccoli have been found to induce increased aryl hydrocarbon hydroxylase (AHH) activity. Subsequently, inducers isolated from these plants were identified as indoles; the principal compounds are indole-3-acetonitrile, indole-3-carbinol, and 3,3′-diindolylmethane. Indole-3-carbinol is a potent inducer of increased AHH, whereas indole-3-acetonitrile is weak in this regard, and 3,3′-diindolylmethane is intermediate in its activity (*1*). Since compounds inducing increased AHH activity can protect against chemical carcinogenesis, work was initiated to determine whether the indoles would do so. When added to the diet, each of the three indoles inhibited neoplasia of the mouse forestomach that resulted from administration of BP. In a second animal model, indole-3-carbinol and 3,3′-diindolylmethane given by oral intubation 20 hours before DMBA inhibited mammary tumor formation in the rat, but indole-3-acetonitrile did not (*2*). In the main text, inhibition of neoplasia by benzyl isothiocyanate, phenethyl isothiocyanate, and benzyl thiocyanate, also constituents of cruciferous vegetables, was described. The presence of an additional group of inhibitors in these vegetables makes them of particular interest.

Cinnamic Acid Derivatives

Cinnamic acid derivatives occur in a wide variety of plants. Two such derivatives, *o*-hydroxycinnamic acid and *p*-hydroxycinnamic acid, have been studied for their effects on BP-induced neoplasia of the forestomach. Both exerted an inhibitory effect (Wattenberg LW: Unpublished observations). A number of carcinogens are inhibited by the synthetic phenols BHA and BHT. The finding of inhibition of chemical carcinogenesis by two naturally occurring phenols adds another chemical class of naturally occurring inhibitors to those already identified and one which potentially could be of considerable importance.

Ascorbic Acid

Although little information is available on inhibition of carcinogenesis by ascorbic acid administered prior to or simultaneously with chemical carcinogens, two reports of such inhibition have been published. In one, ascorbic acid added to the drinking water inhibited neoplasia of the urinary bladders of mice that resulted from implants of cholesterol pellets containing 3-hydroxyanthranilic acid (*3*). In the other, topical application of ascorbic acid to the skin of mice 5 minutes before challenge by topical application of DMBA inhibited initiation of epidermal neoplasia (*4*). In addition, topical application of ascorbic acid (and other antioxidants) to the skin of mice subsequent to initiation by DMBA but during the early period of promotion by Croton oil inhibited epidermal neoplasia (*5*).

The possible inhibitory effects of ascorbic acid on chemical carcinogenesis has taken on additional interest because of mutagenesis studies. Ascorbate inhibits *N*-methyl-*N*-nitrosoguanidine- and dimethylnitrosamine-induced mutagenesis of Salmonella TA-1530 but is ineffective against *N*-methyl-*N*-nitrosourea (*6*). In man, Land and Bruce (*7*) reported that ascorbic acid diminishes the mutagenic activity found in the feces of normal individuals on a western diet. They stated that the mutagens appear to be N-nitroso compounds. A diminished amount of such compounds could result either from ascorbic acid inhibiting formation of N-nitroso compounds (*8*) or reactions occurring subsequent to their formation (*6*).

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GUEST EDITORIAL I. Berenblum, *M.D.*[1]

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Established Principles and Unresolved Problems in Carcinogenesis

Stocktaking in science has a double purpose: *a*) to summarize and evaluate what has already been achieved and *b*) to recognize and define anomalies and problems that are still unresolved. Much more attention is generally paid to the former than to the latter.

From the accumulated information about carcinogenesis [*see* reviews (1–8)], some general principles can be set as a basis for a unified theory of tumor causation, but a number of unresolved problems still appear to conflict with prevailing theory. Although no facile solution is offered here to account for these anomalies, their mere formulation might help one to decide whether the disturbing facts are wrong, their meaning is misunderstood, or the prevailing theory with which they conflict is at fault.

GENERAL PRINCIPLES OF TUMOR CAUSATION

The following include both established principles and those which, while consistent with currently known facts, still lack proof:[2]

1) A tumor is focal in origin, presumably arising from a single neoplastically altered cell; it therefore constitutes a clone (*9*).

2) Carcinogens are capable of acting on a previously normal cell, apparently during the mitotic cycle (*10, 11*), converting it first into a "latent" or "dormant" tumor cell (*12–14*).

3) The overall process of carcinogenesis, with its long latent period, is not a single continuous process but comprises at least two components: *a*) an initiating phase responsible for the conversion of the normal cell into a dormant tumor cell and *b*) a promoting phase responsible for the awakening of the dormant tumor cell, or its progeny, into a progressively growing tumor (*8, 12–17*). However, whether all forms of carcinogenesis involve these two phases is not certain.

4) The initiating phase is brought about rapidly and is presumed to be mutational in mode of action [for alternative theories, *see* (*18, 19*)]. The promoting phase is a slow process and accounts for the long latent period of carcinogenesis, presumably epigenetic in mode of action. It expresses itself, in terms of cell kinetics, as a persistent disturbance in growth equilibrium (*8, 14*).

5) Most carcinogens first have to be metabolized in the body to yield "proximate" or "ultimate" carcinogens, which bind covalently with nucleic acids and proteins in the cell (*5, 20, 21*). Alkylating agents may act without prior metabolic change, combining with DNA and other cell molecules (*22–24*). The reaction with DNA probably occurs during or prior to the S-phase of the mitotic cycle. In the case of complex molecules, their binding with the DNA of the cell presumably leads to chromosome breakage followed by faulty recombination. This might occur during the M-phase of the mitotic cycle (*11*). The possibility of induced mutations undergoing repair (*25*) is now recognized as an important controlling factor in certain types of carcinogenesis.

6) In many instances, the metabolic conversion of a compound into a proximate carcinogen takes place in the very tissue that subsequently undergoes transformation. This is evident from the fact that neoplastic transformation can occur in pure cell culture reacting to the parent carcinogen (*6, 26*). In other instances, where neoplastic transformation cannot apparently be effected in vitro, it is assumed that the metabolic conversion must occur in a remote tissue and the product is then brought to the target organ.

7) The body is also capable of detoxicating carcinogens and converting them into carcinogenically inactive derivatives that are readily excreted (*5*). The relative degree to which a carcinogen is detoxicated or converted into a proximate carcinogen largely determines how effectively, in terms of dose requirements, the parent compound induces tumors (*27, 28*).

8) The metabolism, binding capacity, and other processes involved in promoting action are almost certainly different from the processes involved in initiating action. Whereas initiation most likely involves nucleic acid binding in the nucleus of the cell, promoting action might perhaps be associated with protein binding at the surface of the cell (*29, 30*).

9) The simplest way of relating the action of oncogenic viruses to physical or chemical carcinogenesis is to assume that part of the viral genome becomes incorporated into the genome of the cell either directly, in the case of DNA viruses (*31*), or indirectly, in the case of RNA viruses [with the intermediate step, in the latter

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[2] The latter are expressed with qualifying terms such as "apparently," "presumably," and "probably."

Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

case, of the formation of a DNA provirus by reverse transcription (*32*)]. Thus *added* (neoplastic) information is provided in viral carcinogenesis, as opposed to mutationally *altered* information provided in physical or chemical carcinogenesis.

10) The tumors induced by any particular virus are remarkably alike in their morphology, growth rate, general behavior, and even in their specific antigenicity. The situation is different in the case of tumors induced by physical or chemical agents; no two are identical. The difference is most readily explained by the fact that oncogenic viruses carry very little genetic information, whereas in the case of spontaneous, physical, or chemical mutations the scope for variety is virtually infinite.

11) Many factors are capable of profoundly influencing the animal's response to standard carcinogenic action—either positively (cocarcinogenic) or negatively (anticarcinogenic). The various ways in which these operate (*33*) include: *a*) preparative action on the target organ, *b*) permissive influence relating to the scope of action (e.g., solubility, metabolism, and excretion) of the carcinogen, *c*) "incomplete" carcinogenic action (initiation and promotion), *d*) conditional influences (hormonal or immunologic) on the resulting tumor, and *e*) influence on viral action.

12) The role of hormones in carcinogenesis is somewhat complicated, because apart from their "modifying" role in relation to physical, chemical, and viral action, some hormones seem able to induce tumors by their own independent action, by virtue of a persistent endocrine imbalance in the body. This will be further discussed among the unsolved problems.

EXAMPLES OF THE UNRESOLVED PROBLEMS IN CARCINOGENESIS

1) Strain and Species Differences in Response to Carcinogenic Action

Although genetically determined differences in response to carcinogenic action are to be expected, a surprising lack of parallelism exists, in most cases, between the tendencies toward spontaneous and toward induced tumor development in any particular strain or species. Striking differences also exist in relative organ response to any particular carcinogen among the various strains and species tested.

In general, tissues that respond most readily to local carcinogenic action—skin and subcutaneous tissues—are rare sites for spontaneous tumor development. (Most of the exceptions—e.g., skin tumors in wild cottontail rabbits or subcutaneous tumors in the rabbit or chicken—involve oncogenic viruses and are, therefore, not spontaneous in the genetic sense.)

Admittedly, in instances where spontaneous tumors are rare while responsiveness to extrinsic carcinogenic action is high, absence of the appropriate stimulus rather than insensitiveness of the tissue is the likely explanation. As for differences in response to carcinogenic action among different strains and species of animals, dissimilar rates of metabolism (toward the production of proximate carcinogens) or of catabolism (toward detoxication) are important factors but cannot account for all the inconsistencies.

Why, for instance, should the rat respond well to sc administration of carcinogens but poorly to topical application to the skin, whereas the rabbit responds in reverse, and the mouse responds about equally to both forms of action? The contrast is even more striking in the case of the rabbit, which fails to react to sc administration of so potent a carcinogen as 7,12-dimethylbenz[*a*]anthracene but responds readily to viral action (cf. Shope fibroma and myxomatosis).

Apart from genetic determinants expressed through metabolic, hormonal, or immunologic influences, it has been suggested that the vertical transmission of RNA virus particles [cf. the "oncogene" theory (*34*)], acting in association with chemical carcinogens, might account for some of the strain and species differences in response to extrinsic carcinogenic action. No clear picture has yet emerged from the various attempts to explore these possibilities.

2) Organ Differences in Response to Specific Forms of Carcinogenic Action

In addition to variations in strain and species response, organ distribution of induced tumors varies according to the type of carcinogen used. Why should some carcinogens (notably, the polycyclic aromatic hydrocarbons) cause tumors to develop at the sites of applications in practically every tissue of the body and also act systemically on such tissues as mammary, lung, and hemopoietic, whereas some carcinogens (e.g., 2-acetylaminofluorene, urethan, and nitrosamines) have a restricted range of systemic action and others (e.g., aminoazo compounds, β-naphthylamine, and aflatoxin) are almost exclusively single-organ carcinogens?

The possibility that the distribution of specific enzymes required for the formation of proximate carcinogens might explain this extraordinary diversity in tissue response seems unlikely, inasmuch as the enzymes known to be involved (*5, 20*) do not appear to possess the degree of specificity, or limited distribution, to justify such a conclusion.

3) Carcinogenesis by Hormone Imbalance

Theoretically, a fundamental difference exists between hormonal *influence* in carcinogenesis and hormonal carcinogenesis *per se*, though the distinction cannot always be recognized with certainty in practice.

Hormonal influences on carcinogenesis can operate by: *a*) preparative action on the responding tissue, *b*) permissive influence on the carcinogenic process itself, or *c*) conditional influence on the induced (hormone-dependent) tumor. However, true hormonal carcinogenesis implies that the induced tumor results from hormone imbalance alone. This seems to have been the case in the classic experiment of Biskind and Biskind

(*35*), in which rat ovarian tissue transplanted into the spleen ultimately became neoplastic. The accepted explanation is that persistent, excessive stimulation by the pituitary follicle-stimulatory hormone was responsible for this neoplasia, brought about by interruption of the normal feedback mechanism: The estrogenic hormone, secreted by the transplanted ovarian tissue, had to pass via the portal circulation through the liver where it was destroyed, thus failing to reach the pituitary gland.

The problem here is why overstimulation of a *physiologic* process should bring about so pathologic a change as neoplastic transformation. Such a progression seems to conflict with the notion of a mutational change in the genome of the cell as a prerequisite of neoplasia.

One way of resolving the dilemma would be to postulate that hormones operate merely as modifying factors under all circumstances (i.e., as cocarcinogenic influences in relation to some undetected primary factor) rather than as complete carcinogens.

The following question then arises: What might be the nature of the "undetected primary causative factor"? A spontaneous mutation? A latent oncogenic virus? An unsuspected initiator in the animal's environment? We do not know.

4) Carcinogenesis by sc Implantation of Plastic or Metal Films

The problem here is: How can nonspecific stimuli bring about a mutational change in the genome of the cell? The real conflict is between lack of chemical specificity in the case of plastic or metal films [the only determining factor being the physical state of the film (*36, 37*)] compared with the high degree of chemical specificity in the case of other classes of carcinogens (*7*).

One could again postulate a secondary role and be faced with the same question: Is the "primary causative factor" a spontaneous mutation, a latent oncogenic virus, or an unsuspected initiator in the animal's environment? The available evidence is even more scanty here than in the case of hormonal carcinogenesis.

5) Transformation Efficiency In Vivo and In Vitro

The allegedly striking difference in transformation efficiency between in vivo and in vitro carcinogenesis is not often referred to in the literature. Yet it is an anomaly calling for further inquiry.

Transformation efficiency can be fairly accurately determined in vitro (*38, 39*); under suitable experimental conditions, a relatively high percentage of the cells exposed to a carcinogen can be shown to have undergone neoplastic transformation. Extrapolating these findings to an in vivo system (e.g., in the liver, where millions of cells are exposed to a systemically administered carcinogen), one would have expected hundreds of primary foci of induced neoplasia in that organ. In fact, it is rare to find more than a half dozen such foci, even under extreme conditions of carcinogenic action.

Several explanations for this may be proposed, though none of them seem very convincing:

a) Optimal conditions for carcinogenic action may never occur in vivo when the carcinogen is administered by the systemic route, because it is diluted before reaching the target organ.

b) Enzymatic detoxication of carcinogens may possibly be more efficient in vivo than in vitro, thus accounting for the difference in concentration of the *proximate* carcinogen. (Another factor to be considered is that enzymatic detoxication may not be effective when tissue is oversaturated with a carcinogen.)

c) A high transformation efficiency may actually be reached in vivo but not recognized if most of the transformed cells remain dormant (i.e., not stimulated by effective promoting action). This presupposes that in vitro such additional promoting action is not essential. [For recent examples of the initiation-promotion system operating in vitro, *see* (*40, 41*).]

d) DNA repair following a mutation (*25*) may be more effective in vivo than in vitro.

e) The limiting factor in vivo may be immunosurveillance, an explanation based on the supposition that most neoplastically transformed cells are destroyed by immune processes in the body, with only the occasional tumor cell slipping through the immunologic barrier. This theory (*42, 43*) has since lost much of its popularity, when conflicting evidence began to accumulate (*44–47*).

CONCLUSIONS

The term "anomaly" refers to a situation in which observed facts conflict with prevailing theory. The present "theory" in carcinogenesis is that the initial step in neoplastic transformation constitutes an irreversible change in the genome of the cell. The "facts" that appear to conflict with it are *a*) some of the variations in strain and species response (example 1) and differences in organ response according to the type of carcinogen (example 2), neither of which could have been predicted on theoretical grounds; *b*) the existence of hormonal carcinogenesis (example 3) and sarcoma induction at the sites of sc implantation of plastic or metal films (example 4), neither of which could be thought to act by inducing a gene mutation; and *c*) the apparent difference in transformation efficiency between in vivo and in vitro (example 5), for which no satisfactory explanation is available (assuming that the difference is real and not attributable to some ancillary factor.)

These unaccountable phenomena are stressed in the belief that the solution of the problem of the mechanism of tumor induction might eventually emerge from the indirect approach of resolving existing anomalies, rather than from a more frontal attack of building up plausible theories that fit some of the available facts but ignore exceptions.

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ADDENDUM

As a supplement to the list of references in this Guest Editorial, the following additions dealing with some of the issues raised in the text are offered.

Role of Mutations in Carcinogenesis

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GUEST EDITORIAL

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Immune Surveillance Revisited

THE THEORY OF IMMUNE SURVEILLANCE proposed by Burnet (*1*) and extended by Thomas (*2*) and by Burnet (*3, 4*) postulates that one function of the immune system is to eliminate or prevent the multiplication of nascent malignant cells. This theory has generated much experimental work on a possible relationship between oncogenesis and the immunologic status of the host. Often, results have been contradictory. Studies demonstrating tumor-specific antigens on human and animal tumors, reports of facilitated carcinogenesis by immunosuppression, and clinical observations of the increased incidence of malignancy in immunosuppressed or immune-deficient patients are all cited as evidence in support of immune surveillance (*5–8*). On the other hand, the finding that some tumors do not seem to be antigenic and the failure to demonstrate enhanced carcinogenesis with immunosuppressive treatment in some studies are cited as evidence against the immune surveillance theory (*9–11*). We do not present a case for or against immune surveillance, as this has been done many times in recent years (*9, 12–16*). Instead, we ask whether it is possible to settle this issue at all by either clinical observations or experimental evidence.

Evidence from clinical studies that favors immunosurveillance is twofold: A high frequency of malignancy is observed in patients with various immune deficiency diseases (*17*), and an increased frequency of autochthonous malignancies occurs in immunosuppressed patients with kidney transplants (*18*). However, there are several reasons why immunosuppression (or immunodeficiency) is not the sole explanation for the increased risk of cancer in these patients. For example, whatever caused the immune deficiency disease may be responsible also for the increased susceptibility to cancer. Furthermore, immunodeficient and immunosuppressed patients with transplants are exposed to persistent antigenic stimulation; there is ample evidence from animal models that persistent antigenic stimulation under various conditions can lead to excessive lymphoproliferation and ultimately to lymphomas and reticular cell sarcomas (*19*). An examination of the type of tumors in these patients showed that the excess risk of cancer was largely due to lymphoreticular neoplasms, particularly reticulum-cell sarcomas (*17*, *20*).

The basic premise underlying the experimental studies is that, if immune surveillance exists, its abrogation should result in the development of malignancies in addition to those tumors which would occur in the presence of an intact surveillance system. Attempts to test this hypothesis have been made by determining the effects of immunosuppressive treatment on the development of transplanted syngeneic tumors and autochthonous tumors. Experiments with transplanted tumors have little bearing on the issue of immune surveillance. Once a tumor has arisen in an immunologically intact host, it has already circumvented the immune surveillance system. The mechanical insertion of a relatively large number of preselected tumor cells into a secondary host is in no way analogous to the appearance and growth of a primary autochthonous tumor cell within its unique microenvironment.

In studying the effect of immunosuppressive agents on chemical carcinogenesis, most investigators have looked for increased tumor incidence and growth rate and a decreased latent period. Where such alterations could not be detected, the authors have concluded that immune surveillance was inoperative (*10*, *21*, *22*). However, such negative results do not necessarily contradict the immune surveillance theory. An immune surveillance mechanism would eliminate only tumor clones possessing sufficient antigenicity to be recognized as foreign. If several clones arise at the same time, all must be eliminated to prevent tumor formation. In chemical carcinogenesis, it is not possible to determine whether a tumor arises from the most successful of several competing clones or from a single unopposed clone. However, if many clones originate at the same time, it is likely that at least one will be weakly antigenic and will escape immune surveillance. Under these conditions, the abrogation of immune surveillance might only result in an increased proportion of highly antigenic tumors among those which arise.

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

Many studies have demonstrated that immunosuppressive treatment can potentiate oncogenesis by certain viruses (*15*, *23*, *24*). Although these findings could be interpreted to support the concept of immune surveillance, this is not necessarily true. More likely, these results reflect the abrogation of an immune response against the infecting virus, and they may be unrelated to immune surveillance against autochthonous neoplastic cells [cf. (*14*)].

In addition to obstacles unique to certain experimental systems, 2 general limitations apply to all studies of this type. The first is the inability to detect and measure immune surveillance directly. Instead, one actually measures an immune response. Such a detectable immune response is the net result of an extensive amplification process. Immune surveillance, on the other hand, might function at a cellular level shortly after a neoplastic transformation, and need not involve an amplification mechanism. Agents that interfere with cellular proliferation or differentiation may have a dramatic effect on a detectable immune response but no influence on immune surveillance. For this reason, one can never claim with certainty that the effect of an immunosuppressive agent or procedure on carcinogenesis results from the alteration of an immune surveillance system against autochthonous cancer cells. Conversely, certain agents might abrogate immune surveillance without apparent depression of immunologic responses. For example, appropriate doses of certain carcinogens can depress immune responses (*25*). Even when carcinogen administration does not lead to systemic immunosuppression, a local effect on immune surveillance may prevent the destruction of nascent tumor cells in the area of carcinogen application. If such a phenomenon actually occurs, then the addition of an immunosuppressive agent during chemical carcinogenesis could not be expected to contribute dramatically to tumor induction.

The second limitation in studies of immune surveillance is the inability to abrogate even the measurable immune responses without alteration of other physiologic or homeostatic mechanisms. This is obvious with cytostatic drugs and antimetabolites whose activity is clearly not restricted to lymphoid cells. Even with antilymphocytic serum (ALS), the most selective immunosuppressive agent employed in this context, there may be considerable side effects affecting nonimmunologic processes (*26*). In several studies in which ALS treatment potentiated chemical carcinogenesis, there was evidence that this effect was not directly related to the immunosuppressive properties of the ALS (*27*, *28*). These problems of immunosuppressive treatment have been circumvented by the induction of tumors in vivo in cells confined in a Millipore chamber (*29*, *30*). In this instance, carcinogenesis occurs in the absence of cell-mediated immunologic influences. However, the objection remains that these tumors arise in an environment also devoid of other nonimmunologic regulatory mechanisms. Thus in all experimental systems, it is difficult to attribute the effects of an agent or procedure on oncogenesis solely to its alteration of immunologic function.

What, then, is to be gained from studying the effects of immunosuppressive agents on carcinogenesis? It seems impossible, at present, to design an experiment that will prove or disprove the existence of an immune surveillance mechanism. What we can do is ask a much simpler question: Which agents or procedures are likely to increase the probability of neoplastic disease? We might predict at the outset that, under certain conditions, many "immunosuppressants" will markedly influence neoplastic processes; under other conditions, these agents would be irrelevant to the course of malignancy. A practical benefit from such studies would be to identify the agents capable of augmenting neoplasia and to define the conditions where this is apt to occur. This is especially important because more and more patients will receive long-term immunosuppressive therapy. Current experimental efforts could be directed toward these practical considerations, though they may have no direct bearing on the question of immune surveillance.

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GUEST EDITORIAL

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Considerations of Some Current Concepts in Cancer Research [1]

There are several commonly accepted concepts in cancer research, even though they are still largely unproved. We discuss here two such concepts: 1) immune surveillance, i.e., that neoplastic cells appear frequently in the organism, possess tumor-specific antigens, and are therefore rejected by the T-cell system; and 2) that oncogenic viruses, chemical carcinogens, and physical agents cause cancer either by directly transforming a large number of target cells into neoplastic cells, or, without directly affecting the target cells, by suppressing the immune surveillance mechanism and thereby allowing the spontaneous appearance of potentially neoplastic cells—a common event according to the surveillance concept.

Both these concepts share several features. One is that tumor induction after injection of carcinogenic agents should occur in many cells irrespective of whether the mechanism of carcinogenesis is direct cellular transformation by oncogenic viruses or chemical carcinogens or whether these agents are immunosuppressive and allow the appearance of commonly occurring spontaneous neoplasms. Therefore, the cellular origin of tumors is important for the understanding of the mechanism by which tumors appear spontaneously or after induction.

Monoclonal Origin of Tumors

The first clear evidence of the cellular origin of tumors came from studies on myelomas [for review, *see* (*1*)]. These tumors arise from B lymphocytes capable of synthesizing and releasing immunoglobulins. It was soon found that sera from patients with myelomas contained a typical homogeneous peak of immunoglobulin. All the immunoglobulin belonged to the same class and had identical variable regions, which strongly indicated that myelomas were derived from a single cell. Since each B lymphocyte has unique immunoglobulin receptors as judged by various criteria, the inescapable conclusion is that myelomas always start from one cell. By the use of immunoglobulin markers it has been found that myelomas are not unique in this respect. Various lymphatic tumors have been studied, and monoclonal origin has been proved for Waldenström's macroglobulinemia, chronic lymphatic leukemia, chronic lymphosarcoma leukemia, Burkitt's lymphomas, and other non-Hodgkin's lymphomas. Actually, monoclonality of lymphoid tumors appears to be the rule and few, if any, exceptions are known.

Monoclonal origin of tumors is not restricted to lymphoid neoplasms. However, clonality is easier to study in lymphoid tumors of B-cell origin, because they possess the unique immunoglobulin markers. Another marker, which is less stringent with regard to establishment of clonal origin but has been useful and has clearly indicated monoclonal origin of many nonlymphoid tumors, is the glucose-6-phosphate dehydrogenase marker. This isozyme exists in allelic forms and is X-linked. Therefore, heterozygous women express only one of the two alleles, since only one random X-chromosome is expressed in each cell. Study of whether tumors express one or both alleles can establish monoclonality or polyclonality. In all tumors studied except two, monoclonality has been the rule. At present, 19 different solid tumors, investigated by the use of this marker and found to be of clonal origin (*1*), have been obtained from a large variety of anatomic locations. They have been sarcomas, carcinomas, and various lymphoid tumors; 184 uterine leiomyomas were all monoclonal. Even the common wart, the only clearly virus-induced tumor in humans, shows monoclonal origin, as does Burkitt's lymphoma, another tumor of suspected virus etiology. The only two types of tumors found to be of multicellular origin among all cases studied were hereditary and showed mendelian traits; they are hereditary neurofibroma and hereditary trichoepithelioma.

Monoclonal origin of tumors is not restricted to man. To the same extent as human myelomas, those in animals shows monoclonality as judged by the immunoglobulin markers. These tumors can be induced both chemically and after infection with the Abelson leukemia virus; even when the tumor incidence is markedly increased by the combined injection of a chemical carcinogen and Abelson virus, the myelomas are monoclonal (*2*). There is evidence also that Gross virus-induced T-cell leukemias in AKR mice are monoclonal, although the markers are not as strict as those of immunoglobulin. In addition, Abelson leukemia virus transforms B lymphocytes in various stages of differentiation into neoplastic cells, and each leukemia has a unique pattern of response to various polyclonal B-cell activators, which strongly indicates that neoplastic cells represent a clonal product of one cell transformed at a particular stage of differentiation.[2] Also, chemically and physically induced sarcomas are likely to be clonal. Thus such tumors possess distinct unique tumor-specific transplantation antigens (TSTA) with very little, if any, cross reaction between them. The simplest interpretation of this finding is that the tumor arises from one single neoplastic cell. This is also made likely by studies of Prehn (*3*), who compared the TSTA in opposite poles of the same 3-methylcholanthrene-induced tumors. If the tumor was of multicellular origin, cells from opposite poles would have different antigens, whereas if it was monoclonal they would have the same ones. In nearly all tumors studied, the cells from opposite poles had the same TSTA. The possibility that monoclonality is due to selection or rare variants will be discussed later.

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[1] This editorial continues the discussion initiated with the Guest Editorial of Kripke and Borsos (J Natl Cancer Inst 52:1393–1395, 1974) and continued by Law (Letter to the Editor, J Natl Cancer Inst 54:280–282, 1975).

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Why Are Tumors Clonal?

There are three major possibilities: 1) Carcinogens only transform rare preexisting susceptible variants; 2) a large number of cells become transformed, but only one immunoselected variant becomes established; or 3) carcinogens accelerate the appearance of rare genetic changes leading to neoplasia.

1) The possibility that carcinogens only affect rare susceptible variants is unlikely, whether tumors are induced by chemical carcinogens, physical agents such as X-ray, or RNA oncogenic viruses. Heidelberger (*4*) cloned fibroblasts and, after expanding the clones, showed that all clones were susceptible to chemical carcinogens; this precluded the existence of rare variants. However, within each clone only a small proportion of cells were transformed. Actually, transformation is a rare event in fibroblasts exposed to chemical carcinogens, and all tumors initially appear clonal, i.e., appear to start from one cell. The actual efficiency of carcinogenic transformation varies but is usually low (0.01–1%). Also, with RNA tumor viruses, transformation in vitro clearly is too frequent to allow for selection of rare susceptible variants, but tumor formation is clonal in origin, with a low efficiency of transformation. The only situation where preexistence of susceptible variants may occur is a cytopathic DNA virus such as simian virus 40 (SV40) where the main effect is cytotoxicity of a large number of cells and rare cells appear to be transformed into neoplastic cells.

2) The possibility that immunoselection is responsible for the clonal nature of tumors is unlikely in the case of virus-induced tumors, because these possess the same TSTA, and therefore immunoselection of only one clone is impossible. It could be argued that this mechanism could operate for chemically induced tumors. However, as already pointed out, Prehn's experiments (*3*) comparing TSTA from different poles of the same tumor argue against this possibility. It should also be noted that the possibility of immunoselection, which requires an intact immune system, is incompatible with the immune surveillance mechanism; this suggests that tumors appear because the immune system has failed.

3) The possibility that carcinogens only induce rare genetic changes leading to neoplasia is the one that will be favored in this discussion, and evidence for it will be presented later.

The clonality of tumors indicates a genetic mechanism. A variety of such genetic mechanisms could be visualized, the most simple being a mutation-like event, which, by definition, is rare and should result in monoclonal tumors. Changes leading to a somatic mutation-like event could occur by errors during replication or during repair of damage caused by the carcinogen to the genetic material. The errors during repair could either be a passive event in the sense that the enzymes trying to repair a defect make an error in analogy with a replication error, but they could also be a more active event in which the carcinogen, particularly chemical carcinogen, could actually bind to DNA and initiate an error. The basic ideas to be developed here are analogous to those previously suggested by Burnet (*5*) in relation to a genetic interpretation of aging.

The concept that carcinogens cause tumors by a genetic mechanism implies that mutagenicity and carcinogenicity should occur in parallel. Although this was previously not found to be true, new evidence now strongly favors this view. Thus it has been found that many carcinogens lack mutagenicity, because they have to be metabolized before they can express both carcinogenicity and mutagenicity. Since mutagenicity was usually tested in bacterial systems where this metabolic conversion could not occur, no correlation was found. It is also clear that both chemical carcinogens and physical agents such as UV and X-ray irradiation cause damage to DNA. This will initiate repair, and repair can go wrong. It is well known that individuals with deficient repair, such as those with xeroderma pigmentosum, have a high incidence of skin cancer after UV exposure. This fits the suggested mechanism of carcinogenesis. Fibroblasts from such patients are highly susceptible to carcinogens that damage DNA (*6*). Also, repair of radiation damage is associated with enhancement of malignant transformation (*7*).

Errors during replication should in principle give rise to somatic mutation-like changes, and therefore the mechanism of carcinogenesis would in principle be the same. Division seems to have a role in many carcinogenic events. Thus Heidelberger's first attempt to induce tumors by treating prostate in organ cultures with chemical carcinogens failed entirely (*4*). However, analogous experiments on dispersed cells in culture were successful, and one major difference between organ cultures and dispersed cells in culture is that the latter show a higher rate of division. This is further supported by experiments by Borek and Sach (*8*) using X-rays as well as chemical carcinogens. They found that for neoplasia to appear, one to two divisions were required to "freeze" the carcinogenic change. In that case, both repair and replication may be involved. Certain possible viral carcinogens such as Epstein-Barr (EB) virus seem to have one main effect, i.e., to initiate replication in B lymphocytes. This may actually be the only relevant event for their possible carcinogenicity. The occurrence of replication will by chance cause accumulation of replication errors and eventually the appearance of monoclonal tumors. Heidelberger showed the importance of adding the carcinogen at a certain stage in the division cycle. By synchronizing target cells and using short-lived carcinogens, he demonstrated that the carcinogen must be added immediately before DNA synthesis in order to cause neoplasia (*4*).

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Predictions Based on Conventional Immune Surveillance

1) Monoclonality of tumors is caused by immune selection. As already pointed out, this is unlikely for virus-induced tumors, because they have the same TSTA. Also Prehn's experiments (*3*) on 3-methylcolanthrene-induced sarcomas argue against it. This postulate requires an intact immune system, whereas carcinogens are supposed to be immunosuppressive according to the immune surveillance concept.

2) Monoclonality of tumors does not occur in generally or locally suppressed individuals. This problem has not been studied directly, but appears unlikely for several reasons. The tumors appearing in naturally or induced immunosuppressed individuals are exactly those lymphoid tumors where clonality was first found in normal patients by strict criteria. It is, therefore, expected that this problem will soon be answered. Furthermore, these patients generally develop only one tumor, whereas the immunosurveillance concept implies that multiple tumors in different organs would develop. Also, recurrences in patients treated with immunosuppressive drugs are nearly always regrowth of the original tumor, which strongly suggests clonal origin of tumors in immunosuppressed patients.

3) Clonality should not occur in tumors induced in vitro. All tumors induced in vitro are clonal in origin in the sense that they all start from one cell. Heidelberger clearly established that each clone has its unique tumor-specific antigen, which again suggests clonality (*4*). As already pointed out, DNA viruses such as SV40 probably induce monoclonal tumors also in vitro. The main difference between the tumors induced in vivo and in vitro appears to be that in vivo clonality is the rule with few exceptions, whereas in vitro all start clonal, but the frequency of cells that can be transformed is comparatively high. It has already been suggested that different rates of division in vivo and in vitro may be partly responsible for the comparatively higher incidence of tumor induction in vitro.

4) In vitro-induced tumors should possess, on the average, stronger tumor-specific antigens. This is a consequence of the immune surveillance concept that tumors do not appear normally, because they are normally rejected by the T-cell system. The only chance for tumors to appear is either that the T-cell system is inefficient or that the tumors possess very weak TSTA capable of growing out because they are not efficiently recognized by the T-cell system. In terms of the last possibility, in vitro-induced tumors should generally possess much stronger TSTA. However, direct experiments do not substantiate this conclusion (*4, 9*). Thus the reverse was true in Prehn's experiment (the tumors were less antigenic than those induced in vivo). Heidelberger (*4*), studying the same question, found that the in vitro induced tumors ranged from no antigenicity to expected antigenicity as compared to in vivo-induced tumors. Thus the prediction is not fulfilled.

5) Immunodeficient individuals have an increased frequency of polyclonal tumors. This is an important point in terms of immune surveillance and has been used as an argument for it. It is well known that immunodeficient patients have an increased cancer incidence. For recipients of kidney grafts the frequency is as high as 1%. Also, patients with congenital thymus aplasia have an increased tumor incidence. However, several characteristics of these tumors are unexpected:

a) Immunodeficient patients have a very restricted range of neoplasms. In patients with transplants, up to 60% of the tumors are of lymphoid origin, and this rate is even higher in congenitally T cell-deficient patients. This is not compatible with the immunosurveillance concept stating that neoplastic cells appear normally in the body and are normally rejected. There is, for example, no increase in incidence of mammary carcinoma in immunodeficient patients, although this is the most common tumor in females. The only nonlymphoid tumors showing a slightly increased incidence in patients with transplants are skin, lip, and cervix cancer.[3] This is also true for congenitally thymus-deficient patients.[3] These tumors are all suspected to be of viral origin, and this emphasizes one point to be made in this article, namely that the function of the T-cell system is to counteract virus infection and not to reject newly arising tumor cells. When patients lacking T cells get virus infections they develop the disease caused by the virus, whether a common cold or a malignant tumor.

b) In several diseases with pronounced immunosuppression there is no increased tumor incidence. This is true for leprosy, sarcoidosis, and uremia.[3]

c) Nude mice do not develop spontaneous tumors (*10*), although they lack the T-cell function and are entirely incompetent to reject foreign grafts of any type from any species. These mice should develop a high incidence of spontaneous tumors in terms of the immune surveillance concept, but in a study of 11,000 mice, not a single mouse developed tumors (*10*). This is incompatible with the surveillance concept and in itself constitutes the strongest argument against this concept. It was expected that these mice would get virus infections including those leading to neoplasia, but this did not occur in the study referred to (perhaps due to protected environment used for the mice because of their susceptibility to infection). That nude mice are susceptible to virus-induced tumors has been adequately demonstrated (*11*), and they behave in this sense exactly as newborn mice also lacking a mature T-cell system. This, however, is no argument for immune surveillance, but rather shows that the T-cell system conteracts virus infections. It should be pointed out that antibody-induced, cell-mediated cytotoxicity cannot be used as a surveillance mechanism in nude mice, because TSTA are T cell-dependent antigens. Therefore, 7S antibodies, which are required for this cytotoxic mechanism, cannot be formed in nude mice.

d) Immunosuppressed mice do not develop spontaneous tumors. This has been studied in an extensive series of experiments by Simpson and Nehlsen (*12*). They chose the CBA strain because of its low frequency of spontaneous tumors. Such a low frequency may be due to the fact that CBA mice have an efficient T-cell system rejecting spontaneous tumors. Therefore, if the T-cell system were abolished, the incidence of tumors would dramatically increase. Antilymphocyte serum was used to inactivate the T-cell system; very few spontaneous tumors appeared, the frequency being that of untreated CBA mice. However, the high incidence of tumors in one group were all found to be induced by polyoma virus. This again emphasizes that immune surveillance does not normally operate to prevent spontaneous tumor development but that the main function of the T-cell system is to counteract virus infection. This latter function should not be confused with immune surveillance. There is to date no indication that immunosuppressed mice have an increased incidence of *spontaneous* tumors.

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[3] Melief CM, Schwartz RS: Immunocompetence and malignancy. Submitted for publication.

e) Both immunosuppressed and nude mice are susceptible to viral and chemical oncogenesis. It is important to establish that the T cell-deficient mice used in the studies above were not resistant to carcinogenesis for genetic reasons or because they possessed other surveillance mechanisms. However, the lack of spontaneous tumors when the T-cell system is abolished cannot be ascribed to another surveillance mechanism particular to these mice.

f) Tumors should occur frequently in privileged sites. There are a number of anatomic locations where the T-cell system cannot efficiently operate, such as the anterior chamber of the eye, the cheek pouch of the hamster, and possibly the brain. Therefore, it would be expected that the tumor incidence in these sites would be very high, but this is obviously not so. Again this argues against the surveillance mechanism as the main factor acting to prevent tumor induction.

g) Once the T-cell system has failed, multiple and polyclonal tumors should appear. It is to be expected that when the T-cell system fails (such as in old people) and thus allows the first tumor to appear, it would also have reached such a low efficiency that tumors should appear in other organs or in the same organ as that where the first tumor has occurred, which would make tumors in each organ polyclonal. However, this is not the case. Tumors usually occur only at one site, they are monoclonal, and the frequency of double tumors, is that expected by chance from the frequencies of each tumor type. Furthermore, when patients have been treated with chemotherapy, which is known to be immunosuppresive, the recurrences are nearly always regrowths of the original tumor. This is also true in Burkitt's lymphoma, with its suspected viral etiology.

Predictions Not Based on Immune Surveillance

1) Spontaneous tumors appear infrequently by genetic mechanisms analogous to somatic mutation. It will be argued that this is the main mechanism of tumorigenesis. The mutations appear spontaneously by errors during replication or repair, caused by carcinogen-induced damage to the genetic material. Therefore, carcinogens cause tumor formation, but the mechanism is the same as for spontaneous tumors. Tumor occurrence is a rare event and consequently tumors are expected to be clonal also after experimental induction.

2) The first tumor cells to appear are not rejected by the T cells which have other functions. As already pointed out, evidence suggests that the T-cell function is to defend the individual against virus and other types of microbial infections. If this system fails, the individual becomes susceptible to these infections. In rare cases, people become infected with tumor viruses that induce neoplasms. However, the neoplastic change then, appears to be a genetic one, since the tumors are monoclonal. Even though viruses infect a large number of cells, transformation occurs in very rare target cells. A good example is the EB virus, which can infect all B cells. These B cells become capable of unlimited division and actually most, if not all, established tissue culture lines of lymphocytes in vitro require EB virus infection. In mononucleosis the atypical cells appearing in the blood are T cells, and it has been suggested that they can reject virus-infected B cells. This is likely, since the T-cell function is to counteract virus infection. The T cells can hardly be expected to do that by neutralizing virus particles; rather, they attack and kill virus-infected cells. It is noteworthy that Burkitt's lymphoma is monoclonal in origin and starts at distinct anatomic sites, such as the jaw. Monoclonality shows that EB virus by itself is not sufficient to cause neoplastic changes, since all B cells can be infected by the EB virus. Several mechanisms of neoplastic transformation are plausible. An entirely passive one is the accumulation of errors made possible by a high rate of division of infected cells. It can be speculated that the only B cells that become malignant are those reaching a privileged site, where they are protected from killer T cells and have the ability to proliferate for such a long time that a reasonable chance of error accumulation occurs. This should not be confused with immune surveillance. It is an expression of the normal protective function of T cells against virus infection. Such a mechanism could not operate, e.g., in chemical carcinogenesis. Another mechanism by which virus could cause monoclonal tumors is that the virus may insert itself into the genetic material of only rare cells that previously have acquired some genetic changes or even gross chromosome aberrations (Philadelphia chromosome). The virus may actually code for malignancy in such cells or may initiate replication or repair, which is the actual cause of cancer.

Immunity and Neoplasia

1) TSTA stimulate the immune system when the tumor mass is large enough. This states only that tumor-specific antigens behave like all other antigens. A certain amount of antigenic material is necessary before the immune system is stimulated. Immunologically, it seems unlikely that a single neoplastic cell would be detected by the T cells.

2) The first tumor cells to appear are not recognized or rejected immunologically. Because there is sufficient experimental evidence to support this notion, the "sneak through" phenomenon constitutes the best model for immune surveillance. To cause rejection of a tumor transplant by an immune mechanism, it is necessary to inject a minimum number of tumor cells: A lower number is not rejected but grows progressively and kills the host ("sneak through"). However, the injection of a very low number of tumor cells constitutes the best model for spontaneously appearing tumors and the surveillance concept.

3) Immunologic escape mechanism from immune surveillance requires immunization, as does immune surveillance. Various escape mechanisms have been described to explain how tumors appear despite an efficient T-cell system. The descriptions are based on the fact that T cells are blindfolded either by binding antigen or, more probably, by antigen–antibody complexes. Such complexes might also be formed on the tumor cell surface, making the T cells inefficient to reject it (blocking factors). It is certain, however, that these escape mechanisms require immunization against the tumor. The same type of immunization is required for immune rejection by the T-cell system. T-cell activation is also clearly needed for tumor rejection, since TSTA are known to be thymus dependent. Therefore, the escape from immunologic surveillance does not really explain anything, since escape as well as surveillance require the same immunization. It is also noticeable that the postulated highly efficient surveillance mechanism does not

operate against the strongest known immune system (HL-A) in the case of choriocarcinoma, which represents tumor transplantation across the major histocompatibility system, These cells grow in spite of an immune reaction. If this is due to immunologic escape mechanisms, these obviously have no function in kidney transplantation, where rejection is the universal result across an HL-A barrier in the absence of immunosuppression. Actually, choriocarcinoma illustrates what can be expected from immune reaction against tumors. Despite the strength of the immune reactions, these tumors are malignant unless treated. The other tumor-specific antigens known are much weaker, and it appears unlikely that the T-cell system would be able to reject such neoplasms efficiently.

4) Neoplasia is not particularly prevalent in patients with specific *HL-A* alleles. Several other diseases with a suspected or known immunologic etiology or pathogenesis have been associated with specific *HL-A* genes. This association, by analogy with experimental models, has been thought to depend on the presence of specific *IR* genes, in linkage disequilibrium with specific closely linked *HL-A* genes. Consequently, if *IR* gene-regulated T-cell function (of immune surveillance type) should be important in the etiology of cancer, it might be expected that various cancer forms are linked to specific *HL-A* alleles. However, neoplasms so far constitute a disease group in which no such linkage has been statistically documented. If viruses have a function in neoplasia, such a linkage might be expected due to a possible *IR* gene-determined response to a particular virus.

5) Hereditary neoplasms are polyclonal in patients with an intact immune system. This important finding (*1*) shows that the only polyclonal tumors known appear in individuals with an intact immune system. These tumors, however, have a simple heredity, strongly favoring the genetic cause of cancer. It has been suggested that these patients have an initial genetic change necessary for the establishment of cancer, and if this is followed by a second or possibly more genetic changes, tumor cells will appear. Since the chance of acquiring a second change is much increased if all targets already have the first necessary change, it is likely that the tumors will be polyclonal. In normal individuals the chance of acquiring two or more genetic changes analogous to somatic mutation in an individual cell is very small. It is likely that gross chromosome abnormalities function as a first genetic change, making it easier for further mutations to cause neoplasia. This may be the basis for the appearance of the Philadelphia chromosomes in leukemia patients, for the chromosome changes found in patients with Burkitt's lymphoma, or for the increased cancer incidence in individuals with chromosome aberrations such as in Fanconi's syndrome or Klinefelter's syndrome. In all these instances, the gross chromosome change may constitute the first step toward neoplasia on which subsequent mutation works, or these changed chromosome regions may allow the insertion of viruses which, either by damaging the genetic material and initiating repair or by their coding for neoplasia, could cause the appearance of cancer. In the latter instance, it is to be expected that the virus selects only a rare cell with a chromosome abnormality, thereby causing the appearance of monoclonal tumors characterized by certain chromosome markers. Also, rare genetic changes possibly occur in stem cells, which allow the progeny of these stem cells to have an increased susceptibility to viral or chemical carcinogens by mechanisms discussed above. This may be the case with the Philadelphia chromosome and abnormalities in myeloid leukemia, in which not only the tumor, but also normal cells have the same abnormality.

Consequences and Conclusions [4]

1) Viral, chemical, and physical carcinogens cause the appearance of tumors by damaging the genetic material, increasing the replication rate, or allowing insertion of oncogenic virus into changed DNA regions. 2) Hereditary changes may increase the chance of neoplastic transformation by being the first of several necessary genetic changes. 3) There is no need for immune surveillance. 4) There is no immune surveillance. 5) There is no selection against tumors, because they are rare and appear after reproductive age.

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[4] No attempt has been made to review all relevant literature. For detailed references, *see* recent review articles (*13–20*).

ADDENDUM

There have been few experimental or clinical findings since the publication of our original article that expressed our support of the concept of immunologic surveillance against neoplasia. It has become increasingly clear that spontaneous tumors in mice often lack detectable tumor-specific antigen, and therefore it is unlikely that immune surveillance can operate when this occurs.

The finding that nude mice do not develop spontaneous tumors or do so to the same extent as their normal littermates (*1*) still constitutes a strong argument against immune surveillance. It has been suggested that natural killer cells present in nude mice are responsible for immune surveillance. Since tumors of all types can readily be transplanted in nude mice, this suggestion implies that natural killer cells can effectively remove only those spontaneous tumors arising within the individual but cannot reject transplanted tumors. This appears to be a highly unlikely possibility without preference in the literature.

The only instance when immune surveillance may operate is in virus-induced tumors, since T-cells appear uniquely competent to kill virus-infected cells. How often tumors are caused by viruses in nature remains to be determined. Presently, few examples of horizontally spread virus infections causing cancer are available in animal systems and, in these examples, the immune system fails to prevent tumor induction. However, the lymphoid tumors appearing in patients who received transplants may represent virus-induced cells, which differ from other lymphoid tumors by the fact that they are not monoclonal (Simmons RL: Personal communication). Since several viruses such as Epstein-Barr and herpes simplex are known to act as polyclonal B-cell activators and cause transformation of resting B-cells into antibody-producing and progressively growing cells, virus infection in the absence of T-cells may be responsible for these conditions. Obviously, natural killer cells failed to prevent the appearance of tumors in these patients.

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GUEST EDITORIAL by Harry L. Ioachim, *M.D.* [1, 2]

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The Stromal Reaction of Tumors: An Expression of Immune Surveillance

The rise and fall of concepts in science are a part of the evolution and progress of knowledge; however, some ideas have a short, meteoric life whereas others persist for a long time, enjoy a broad-based support, and prove to be remarkably resistant to challenge. Not unlike other candidates for public favor, to be successful and popular, concepts have to be attractive, satisfy our scrutiny, and capture our imagination. The immune surveillance of tumors has proved to be such a concept with *charisma* (*1, 2*); this idea registered an early success, was widely accepted, and efficiently induced a large amount of research.

Thus far the challenges to the Thomas-Burnet hypothesis have been few and were repulsed with relative ease. When the immune surveillance concept was last reviewed and assessed in a conference (*3*), most of the participants agreed that it was still effective as a working hypothesis and that some apparent inconsistencies were not entirely irreconcilable (*4*). In the words of one conferee (*5*), the existence of a surveillance mechanism in cancer "had already been canonized." However, concepts are not allowed to live a peaceful life, and now, a few years later, new heretics have appeared to challenge the canons (*6–10*).

This editorial presents morphologic evidence supporting the concept of immune surveillance. Surprisingly, this line of evidence, possibly one of the strongest yet easiest to ascertain, has been until now largely neglected. However, before discussing the morphologic expression of immune surveillance, perhaps we could discuss a few theoretical objections raised by the latest nonbelievers of the concept.

POINT-COUNTERPOINT

In a recent guest editorial in the Journal, Möller and Möller (*9*), reviewing the immune surveillance of tumors, discussed a series of objections that would disprove the validity of this concept. One objection is that cancer patients show no increased risk of developing a second tumor, although their demonstrated failure in immune surveillance would make it expectable: "Once the T-cell system has failed, multiple and polyclonal tumors should appear. It is to be expected that when the T-cell system fails (such as in old people) and thus allows the first tumor to appear, it would also have reached such a low efficiency that tumors should appear in other organs or in the same organ as that where the first tumor has occurred, which would make tumors in each organ polyclonal. However, this is not the case. Tumors usually occur only at one site, they are monoclonal, and the frequency of double tumors is that expected by chance from the frequencies of each tumor type."

However, this objection apparently stems from misinformation because at an International Conference on "Multiple Primary Malignant Tumours" held in Perugia, Italy, in June 1973, speakers from different countries documented the occurrence statistically. The following are some data quoted from the numerous papers presented:

a) A study reviewing 158,000 cancer patients in Connecticut over 30 years revealed 5,000 cases of multiple primary tumors (*11*). Over 80% of the patients with 2 primary tumors had both tumors microscopically confirmed as independent malignant neoplasms. When the results were analyzed, it was concluded that patients with one malignant neoplasm had 1.29 times the risk of developing a new independent primary tumor than did individuals who never had cancer ($P<0.1$).

b) In a study from Japan (*12*) of 61,869 malignancies, 967 were cases of multiple cancers with the peak incidence in patients 60–65 years of age.

c) A study from the Boston City Hospital (*13*) reviewed 10,977 consecutive autopsies that disclosed the presence of cancer in 2,734 patients. The 3,008 malignant tumors (exclusive of skin and lips) represented 1.1 malignancies per patient. Thus more than 9% of this large series of autopsies on cancer patients revealed more than one primary malignancy. Of the 249 patients with multiple cancer, 235 (94%) had 2 tumors and 14 (5.6%) had 3 or 4 tumors.

d) A study from the M. D. Anderson Hospital and Tumor Institute in Houston (*14*) involving 5 million persons for 25 years concluded that the incidence rate of second primaries in cancers of the skin is at least 140 times higher than that of first primaries, and that, with an incidence of total cancer (except for skin tumors of exposed areas that approached 300 per 100,000) an incidence rate of 25 cancers per 100,000 persons may be expected of second and subsequent primaries.

ABBREVIATION USED: HPS=hematoxylin–phloxine–saffranine.

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

Reviewing the theory of immune surveillance of tumors, some authors contend that predictions based on this concept are not borne out in reality and, therefore, disprove the concept. However, examination of the predictions reveals that some of them were not well founded and perhaps should not have been made in the first place. For example, it is said that (*9*):

"Tumors should occur frequently in privileged sites. There are a number of anatomic locations where the T-cell system cannot efficiently operate, such as the anterior chamber of the eye, the cheek pouch of the hamster, and possibly the brain. Therefore, it would be expected that the tumor incidence in these sites would be very high, but this is obviously not so. Again this argues against the surveillance mechanism as the main factor acting to prevent tumor induction."

This obviously theoretical argument ignores the basic facts of the examples taken for its support. Tumors arise from cells, and a glance through any textbook of histology would show that scarcely any cells are found in or around the anterior chamber of the eye. Tumors also require high rates of cell multiplication for their genesis, a point made by the critics themselves in their review (*9*), yet it is well known that the mitotic rates in the adult brain are very low to nil.

Like these last examples, other objections raised by the critics of the theory of immune surveillance have in common a basic error of logical reasoning by which one factor is singled out of a complex of interreacting causative factors, examined for its conformity with the hypothesis under consideration, then rejected as unfitting along with the hypothesis. Such an objection is the following (*9*):

"In several diseases with pronounced immunosuppression there is no increased tumor incidence. This is true for leprosy, sarcoidosis, and uremia."

The oversimplification of facts is obvious in this argument that does not take into account essential factors such as a uremic patient's life-span, which is too short to allow the slow process of tumorigenesis to develop. And then, of course, one does not know if any oncogenic agents are operative in individuals with leprosy, sarcoidosis, or uremia, since it should not be forgotten that immune surveillance is not involved in the induction of tumors but only in their control.

Another example of a complex phenomenon that is only unilaterally analyzed is that of the growth of choriocarcinomas (*9*). These malignant tumors, although they express strong HL-A antigens, are characterized by rapid growth and invasion, in contrast to kidney transplants in which the expression of similar histocompatibility antigens accounts for their invariable rejection. But here, as in other situations, the immune surveillance may not be the only mechanism responsible for the growth or abortion of a tumor. The successful growth of choriocarcinomas against histoincompatibility barriers is probably related to still unknown shielding functions that act during pregnancy and facilitate the growth of the fetus against the same protective barriers (*10, 15, 16*). In addition, the "sneaking through" phenomenon observed by Old et al. (*17*) and alluded to by other reviewers (*10, 18, 19*) may help to explain why the transplant of a whole kidney is not as easily accepted as the insidious growth of a few tumor cells. By the same token, a transplantable tumor composed of masses of preselected tumor cells is not analogous to the first autochthonous tumor cells that arise in the incipient phase of a tumor (*20*).

A favorite argument of most opponents of the surveillance theory (*8–10*) is that of the low incidence of spontaneous tumors in athymic (nude) mice. According to the predictions (*9*), these mice that lack the T-cell function and are incompetent to reject heterografts should develop a high incidence of tumors, but in a study of 11,000 nude mice (*21*) not a single mouse developed tumors. The argument appears decisive; however, closer scrutiny indicates that the incidence of spontaneous tumors in nude mice was evaluated on populations of animals that lived only an average of 4 months due to their high susceptibility to various infections (*21*). In contrast, a more recent study (*22*) shows that nude mice maintained under strict germfree conditions lived for as long as 2 years and developed during this long life-span many tumors. The average age of tumor occurrence was 14 months. Thus when all possible factors are taken into account, a strong argument against tumor surveillance is turned into new evidence supporting this concept.

Evidently, the strongest argument for the existence of a relationship between tumorigenesis and immunity is furnished by the high incidence of cancer in immunodeficient individuals (*23, 24*). A review of the literature (*8*) shows that neoplasia develops in about 10% of individuals with spontaneous immunodeficiency diseases, whereas a recent report (*25*) records a 5–6% incidence of de novo malignant neoplasms in immunosuppressed organ homograft recipients (a frequency 100 times greater than that in the corresponding general population). Of this very high number of unexpected tumors, most are lymphoid neoplasms, which is taken by some authors (*8, 9, 19*) to indicate the inadequacy of the immune surveillance hypothesis.

Some critics argue that, according to predictions based on immune surveillance, a wide range of tumors with the usual organ distributions should be expected to arise in the absence of control mechanisms (*8, 9*). Lymphoreticular neoplasms were 700 times more frequent than expected (*26*) in female recipients of renal allografts, yet breast cancer (the most common tumor in women) showed no significant increase (*8, 9*).

Here again, a complex multifactorial process appears to be oversimplified for the sake of the discussion at hand. If mammary or other types of carcinomas do not occur more often in immunodeficient individuals,[3] more than one cause may be operative. Breast cancer is possibly the result of an interaction between a hypothetical virus, unknown genetic factors, and an imbalance of hormone homeostasis. Not all of these etiologic factors may be present in immunodeficient patients. However, such complex mechanisms probably do not even have to be invoked in this instance because a simpler explanation is readily apparent.

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[3] This notion may also change in the future. Recently published data (*25*) showed 166 new malignancies occurring in 160 patients treated with chemotherapy. More than a third were epithelial tumors representing 16 different organs.

Carcinomas of the breast or any other organs have long latency periods extending from the time of their induction to the time of their clinical diagnosis. Periodic examination of smears and biopsy specimens of patients with dysplastic lesions of the bronchi and uterine cervix shows that such changes take years to progress to the stage of carcinoma in situ and subsequently to that of invasive carcinoma. Similarly, we have learned, through the recent discovery of vaginal adenocarcinomas developing as a result of maternal intake of diethylstilbestrol during pregnancy, that the latency period for these tumors is well in excess of 18 years (*27*). If immunosuppressed and immunodeficient patients were to live long enough, perhaps breast and other carcinomas would eventually develop in proportions similar to those in the general population. But these patients, just like the athymic mice, succumb earlier to intercurrent infections and lymphomas. Here again, the similarity with the nude mouse is striking because in both immunodeficient mice and man, lymphoid tumors are by far the most common neoplasms. However, this event should not be surprising but expected. Concomitant immune suppression and stimulation have been shown to be strong inducers of neoplasia (*28–30*). In its effort to compensate immunodeficiency, the lymphoid system is stimulated to unusually high rates of proliferation, thus fulfilling one major prerequisite for neoplasia. Corroborating this is the upset of immune homeostasis, which is expressed by imbalances between T- and B-cell systems and results in erratic immune responses with the occasional induction of neoplasia. This sequence of events evokes the classic experiments of Biskind and Biskind (*31*) and Furth et al. (*32*) that demonstrate the consistent occurrence of neoplasms in hyperstimulated target organs after the disruption of endocrine feedback mechanisms (*33*).

In general, when the merits of immune surveillance are discussed, critics of this concept give too much consideration to the ultimate successes or failures of this system, unfairly equating its effectiveness with its existence. Yet the assessment of the effectiveness of the immune surveillance system, not unlike the assessment of the effectiveness of any intelligence agency, is a difficult task because only the failures are evident while the successes remain inapparent. In addition, more consideration should be given to the highly efficient devices of escape by which tumor cells can elude the immune response and their eventual annihilation (*18, 34*). Antigenic modulation (*35*), one of the most subtle mechanisms of cellular adaptation, has been only partially explored. However, it has been demonstrated that by various experimental manipulations, strongly antigenic tumor cells can be made to lose their antigenicity with a consequent increase in transplantability from 0 to 100% (*36–40*).

Finally, in some discussions that review the merits of the theory of immune surveillance, a major distinction is made between virally and chemically or otherwise induced tumors. It is claimed that the T-cell system operates protectively against viruses whether they cause "a common cold or a malignant tumor" and thus prevents carcinogenesis, whereas no similar mechanism is in effect against tumors induced by other agents (*9*).

Although the first part of this assertion might be correct, it must be added that T-cells are operative not only against oncogenic viruses but also against neoplastic cells transformed by viruses; thus they perform the essential function of immune surveillance of tumors.

To assess the immune response to tumors "not induced by viruses" is substantially more difficult because such a category is ill defined if not nonexistent. The apparent difficulties encountered by the immune systems in dealing with such tumors may be explained according to George Klein (*18*) by the more recent appearance of chemical carcinogens in nature. He also made the pertinent observation that several oncogenic viruses (*Herpesvirus saimiri, H. ateles,* polyoma) rarely induce tumors in their host of origin; this suggests that protective immune mechanisms may have evolved over long periods of time and been genetically transmitted by immune responsiveness genes (*18*).

A few years ago, Prehn (*28*) noted that attempts to further demonstrate a theory as well established as that of immune surveillance may become boring. What the theory needs now is to be refined and developed by the incorporation of new facts that have come along, some of them at apparent odds with the theory itself.

Hypothetical mechanisms such as immunostimulation (*28*) or dysfunction of immune homeostasis are complementary rather than contradictory to the concept of immune surveillance and can be certainly included in a broader theory.

In the second part of this paper, I would like to present what seems to be a neglected aspect of immune surveillance against tumorigenesis.

THE STROMAL REACTION OF TUMORS

Pathologists have long been aware that proliferating tumor cells are accompanied by infiltrates of lymphocytes and have regarded this as an indication of host reaction to tumor invasion. Russel in 1908 (*41*) coined the term "stroma reaction" to designate this occurrence, and Murphy in 1921 (*42*) introduced the idea that "immunity to cancer whether natural or induced is attended by lymphoid stimulation." Subsequently, many authors attempted to establish a correlation between the inflammatory reaction of tumors and their prognosis (*43–48*), whereas others, disappointed by inconsistent results, rejected the concept entirely (*49–51*).

In at least two forms of cancer—medullary carcinoma of the breast (*52*) and Hodgkin's disease (*53*)—a positive correlation between the presence of large lymphocytic infiltrates and favorable prognosis is well recognized. However, attempts to directly correlate the local tissue reaction of various cancers with complex events such as recurrence of tumor or survival of patients are rarely successful because in these oversimplified comparisons important clinical parameters are disregarded. Perhaps this explains why the concept of stromal reaction, which preceded by half a century the modern theories of cellular immunity to cancer, is not used for practical purposes today. Pathologists seldom mention in their reports the reactive cellular infiltration of tumors and no accepted criteria exist for its evaluation. On the other hand, tumor immunologists study the cellular immunity to cancer mostly in systems in vitro and are not familiar with the histologic appearance of stromal reactions. In addition, most studies investigate the function not of lymphocytes from the tumor site (which are more likely to be primed by tumor antigens) but those from the peripheral blood, which may or may not include members of the specifically sensitized population of cells.

Although the assessment of cellular immunity to tumors may be elusive, its presence is easily ascertained by simple histologic observation.

The nests and cords of invasive carcinomas of various organs are frequently surrounded by agglomerates of lymphocytes. In such sections, the dense aggregation of lymphocytes around tumor cells that contrasts sharply with their complete absence in the adjacent tissues (figs. 1, 3), is hard to explain other than by specific cellular attraction. Another aspect frequently seen is the invasion of tumor tissue by large amounts of lymphocytes and occasionally by plasma cells. Sometimes, as seen in squamous cell carcinomas of the lung and uterine cervix or in adenocarcinomas of the stomach and colon, the cell infiltrate may even exceed the amount of tumor tissue. The lymphocytes penetrate between and into tumor cells (fig. 7) and display various cell interrelationships including those previously described in tissue cultures as *emperipolesis* (*54*). Electron microscopically, the lymphocytes and macrophages appear to be in intimate contact with the tumor cells and frequently exhibit interdigitating processes (*55*). As a result, tumor cell death is commonly observed.

Considering that the cellular stromal reaction is an expression of immune surveillance of neoplasia, we investigated its presence in the early rather than in the late stages of tumor growth. We were thus able to note the occurrence of heavy lymphocytic aggregates bordering the foci of various carcinomas in situ. In such neoplastic processes as carcinoma in situ of the bronchial epithelium, uterine cervix, and vaginal mucosa, intraductal mammary carcinoma (fig. 2), Bowen's disease of the skin (fig. 3), and Paget's disease of the nipple (fig. 4), although the tumor cells have not crossed the basement membranes and are still strictly intraepithelial, large collections of lymphocytes usually border the lesions and separate the epithelial cancer from the underlying stroma. Even when the epithelial changes are only preneoplastic (such as those of dysplasia of laryngeal, bronchial, or cervical epithelia), numerous lymphocytes are selectively aggregated below the affected areas. In superficially spreading melanomas (figs. 5, 6) as well as in incipient basal cell carcinomas of the skin, the accumulation of lymphocytes consistently accompanies the tumor, and in doubtful instances furnishes a reliable indicator of neoplastic change.

The histology of precancerous and early cancerous lesions compared with that of advanced, invasive, recurrent, or metastatic carcinomas almost invariably shows a significantly greater cellular stromal reaction in the first group of neoplasms (*56*). This observation is not a contradiction but rather is in accordance with the concept of immune surveillance of tumors. Indeed, a more vigorous and probably more efficient cellular reaction is to be expected in the early stages of tumor growth, when the antigenicity of newly transformed cells may be fully expressed and the reactive capacity of the host may be stronger, than in the late stages when the least antigenic clones of tumor cells may have been selected and the immune reaction of the host has been already overwhelmed. Strong support for the preceding interpretation is again provided by microscopic studies of tumor tissues. Pathologists have reported on more than one occasion the spontaneous regression of various tumors. This phenomenon is not uncommon in malignant melanomas, in which the partial or total disappearance of primary tumors has been clearly documented (*57*, *58*). More pertinent to the present discussion is that the tumor regression is constantly accompanied by a heavy lymphocytic infiltrate. Surrounded by lymphocytes and phagocytes, the tumor cells disintegrate and the entire area is eventually replaced by fibrous tissue. By the time the primary tumor has entirely healed, metastases may have already occurred; this provides an explanation for the curious event of metastases without primary tumor, estimated to occur in as many as 8% of malignant melanomas (*59*).

In a recent study (*60*), we examined the cell infiltration at the tumor site in 50 lung carcinomas using a set of histologic criteria designed to permit quantification and comparison. We were thus able to recognize patterns of cell reaction that were characteristic for different histologic types of lung carcinoma and that seemed to be applicable to tumors of other tissues. The amount of cell infiltration was greatest in squamous cell carcinomas (fig. 7) and lowest or nonexistent in oat cell carcinomas (fig. 8). Within the various histologic categories, the well-differentiated carcinomas appeared to be accompanied by more reactive cells than did the poorly differentiated carcinomas.

The plasma cells were distinctively associated with squamous cell carcinomas, and the number of plasma cells in the stroma of such tumors was proportionate to the degree of differentiation and the presence of keratin produced by the neoplastic cells.

The association between plasma cells and squamous cell carcinoma was confirmed by a subsequent study (manuscript in preparation) in which we examined squamous cell carcinomas of other organs including tongue, esophagus, cervix, endometrium, anus, and skin. Not infrequently, the lymph nodes draining the tumors and including metastatic squamous cell carcinoma were also populated by masses of plasma cells replacing the normal population of lymphocytes (fig. 9).

In the search for a biologic meaning to these correlations, it appears that lymphocytes and plasma cells accumulate in and around tumor tissue in response to specific antigenic stimulation. This is not a nonspecific reaction to infected or necrotic tissues as frequently assumed, because *a*) the cells responding to infected or necrotic tissues are invariably polymorphonuclear leukocytes, not lymphocytes and plasma cells; and *b*) the carcinomas with the greatest amount of necrosis (oat cell) have the least amount of cell infiltration. The correlation between degree of tumor differentiation and amount of cell infiltrate further strengthens the assumption that the reactive cells are specifically attracted to the tumor site by tumor-associated antigens. Since undifferentiated carcinomas are accompanied by minimal cell infiltrates, it may be assumed that such tumor cells are less antigenic. During the progression of tumors, as a result of clonal selection, new cancer cells possibly emerge that are poorly differentiated and exhibit little or no antigenic expression. Therefore, such cells are more likely to escape immunologic recognition and annihilation and to give rise to cancers of increased resistance and aggressiveness.

The large numbers of plasma cells and lymphocytes present in the tumor stroma and their apparently selective association with different tumor types suggest that tumor-specific antibodies are released by these cells and that the tumor site is the logical place to attempt their recovery. This assumption is supported by the finding that most cells that infiltrate the tumor stroma exhibit strong immunofluorescence when reacted with fluorescein-labeled antiserum to human immunoglobulins (fig. 10). Further evidence that lymphoid cells at the tumor site release tumor-associated antibodies comes from recent studies showing that eluates of both experimental (*61*) and human (*62–64*) tumors contain significant amounts of immunoglobulins. In most instances these represent tumor-specific antibodies that, under certain conditions, may block the lymphocyte-mediated cytotoxicity in vitro (*62, 65*) and possibly in vivo (*61*). Our results with immunoglobulins recovered from solid tumors and pleural effusions in lung carcinomas (*60*) and from peritoneal effusions in ovarian carcinomas (*66, 67*) have shown that such immunoglobulins react specifically in indirect immunofluorescence with lung and ovarian carcinoma cells, respectively, but not with normal lung and ovarian cells or with cells of various other tumors. These results further strengthen our assumption that populations of lymphocytes and plasma cells aggregate at the tumor site in response to the neoplastic growth and are actively engaged in immune reactions.

CONCLUSIONS

1) Infiltrates of lymphoid cells are present at the tumor site in most neoplasms, predominantly and sometimes exclusively in the early stages of tumor growth. In some neoplasms, the lymphoid infiltrates are associated with tumor regression.

2) The amount of cellular infiltrate usually parallels the histologic grade of tumor differentiation.

3) Distinctive patterns of stromal cellular reaction correlate with different histologic types of neoplasms.

4) In pulmonary tumors, the amount of lymphoid infiltration is highest in squamous cell carcinoma and lowest or nonexistent in oat cell carcinoma.

5) Plasma cells are consistently and characteristically associated with squamous cell carcinomas of various organs.

6) Cellular infiltrates at the tumor site comprise significant amounts of immunoglobulins of all classes. Immunoglobulins with specific reactivity can be recovered from neoplastic tissues and effusions.

7) Lymphocytes and antibodies with specific, tumor-associated activity are more likely to be concentrated at the tumor site; this suggests that for their isolation and identification, tumor tissues are preferable to peripheral blood.

8) The concept of immune surveillance of tumors is alive and well, since none of the criticisms raised thus far has inflicted any mortal wound. New facts and concepts may be included to broaden its scope and our understanding.

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ADDENDUM

Only 2 years have passed since the writing of this Editorial, a short interval in the life of biologic concepts. However, with the unprecedented amount of new information constantly being published, biologic concepts can gain or lose ground and general trends can be discerned from time to time. In the matter of immune surveillance of cancer, concern was expressed at that time that some aspects of tumor growth are not explainable by this concept and may even constitute strong arguments against its validity.

In this respect, new data show that the incidence of neoplasia increases significantly in immunosuppressed recipients of transplanted organs. Their risk of developing cancer is 5 to 6% which is 100 times higher than that of the general population in the same age range (*1*). Sometimes, the tumors originated in the transplanted organ, which was the site of undiagnosed cancer. Under normal circumstances, tumor transplants in unrelated human recipients are practically impossible to achieve, and the high number of cancers (35%) developing in recipients of organs from donors with cancer (*1*) suggests a definite failure in immune surveillance.

The tumors developing de novo showed a steady yearly increase in incidence, e.g., 3% at 1 year, 25% at 5 years in cardiac transplants (*2*); and 11% at 1 year, 24% at 5 years in renal transplants (*3*). The age of cancer patients is younger (*1*) and the multicentricity of tumors is higher in transplant recipients than in the general population (*1, 3*). The time of tumor appearance after an organ homograft is shorter (average 34 mo) (*1*) than the intervals of 15 to 20 years usually recorded for neoplasms arising in X-irradiated patients. The organ distribution of tumors which included mostly lymphoid organs in the first cases reported, a situation taken by some authors to indicate the inadequacy of the immune surveillance hypothesis (*4, 5*), has widened considerably, now comprising almost all tissues and organs. However, a characteristic spectrum of organ involvement is emerging, showing remarkable similarities in various studies.

Tumors of the skin in transplant recipients are the most common, and squamous cell carcinomas occur more frequently than basal cell carcinomas (*1, 3, 6*), contrary to the distribution of skin tumors in the general population. An unusually high incidence of brain tumors, particularly reticulum cell sarcomas, are reported, but leukemias are more often of myeloid type and Hodgkin's disease is infrequent (*1*).

More interesting and distressing is the high malignancy and the propensity for metastasis observed in the tumors occurring in transplant recipients, even in histologic types such as cutaneous squamous cell carcinomas (*1, 3*) commonly known to exhibit a local and protracted course. Finally, at the time of our Editorial, a point was made by one critic of immune surveillance that no increased tumor incidence had been observed in diseases with pronounced immunosuppression such as uremia (*5*). Our counterpoint was that perhaps uremic patients do not live long enough to develop tumors. This supposition appears to apply correctly to the time before the advent of hemodialysis because a recent paper reported (*7*) that, in patients now living longer due to this treatment, numerous tumors have developed. In 646 uremic patients, not under immunosuppressive treatment, the tumor incidence was 2.2%, seven times higher than in the age-matched general population; other studies report even greater incidence figures. The cancers were of a broad spectrum, closer to that of the population-at-large rather than to that of transplant recipients, perhaps because in the latter group immunostimulation due to the allograft acts in addition to immunodepression and thus affects different organs and tissues.

The data quoted in this short updating might be interpreted differently by different people. In view of this writer, they are best understood in the framework of the immune surveillance theory, not in its most simplified form but in a complex interrelationship that takes into account the multiple factors of immune deficiency and immune stimulation that have a critical role in tumorigenesis.

In the second part of the Editorial, I expressed the idea that the site of tumors includes in a concentrated form the activities of cellular and humoral immunity and is therefore a preferential location for their study. Similar ideas formulated by other authors gained momentum, and the in situ expressions of antitumor immunity were recently examined by various experts who attended an international symposium on this subject (*8*). We recently reported the application of these concepts to the study of cellular (*9*) and humoral (*10, 11*) immunity in human lung cancer; further investigations regarding tumors of other organs are in progress.

Without doubt, our knowledge of tumor immune regulation is only in its incipient stage, and many more updatings of our concepts will be necessary before any clear understanding of these complex mechanisms can emerge.

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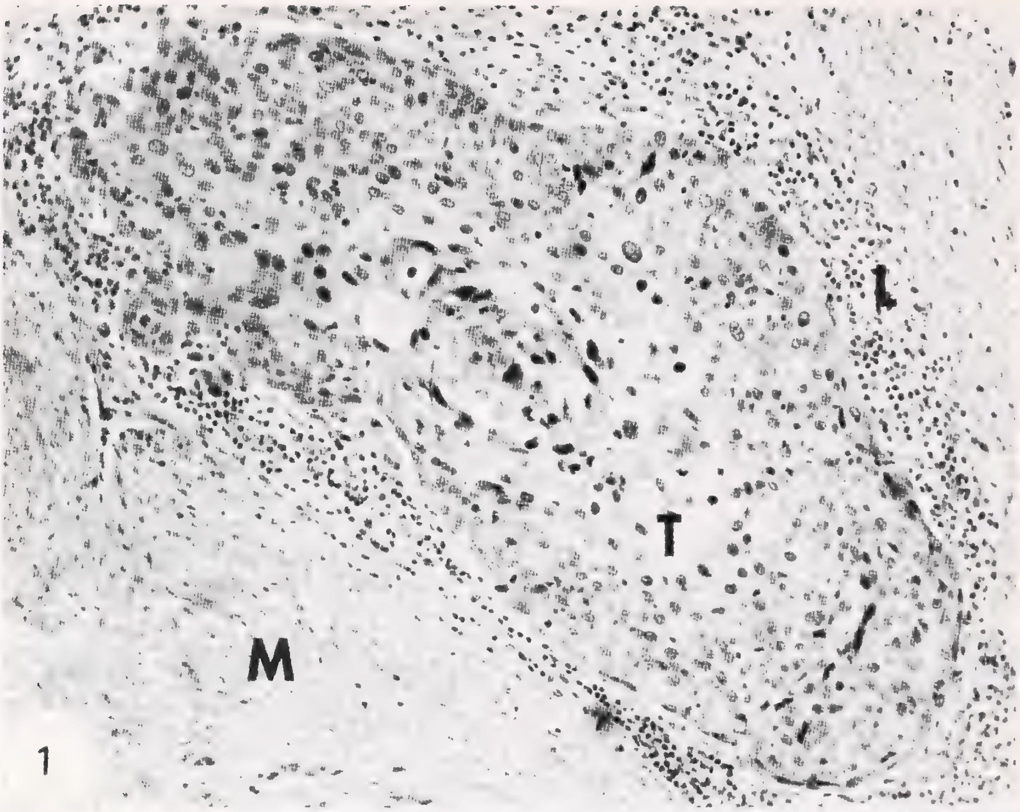


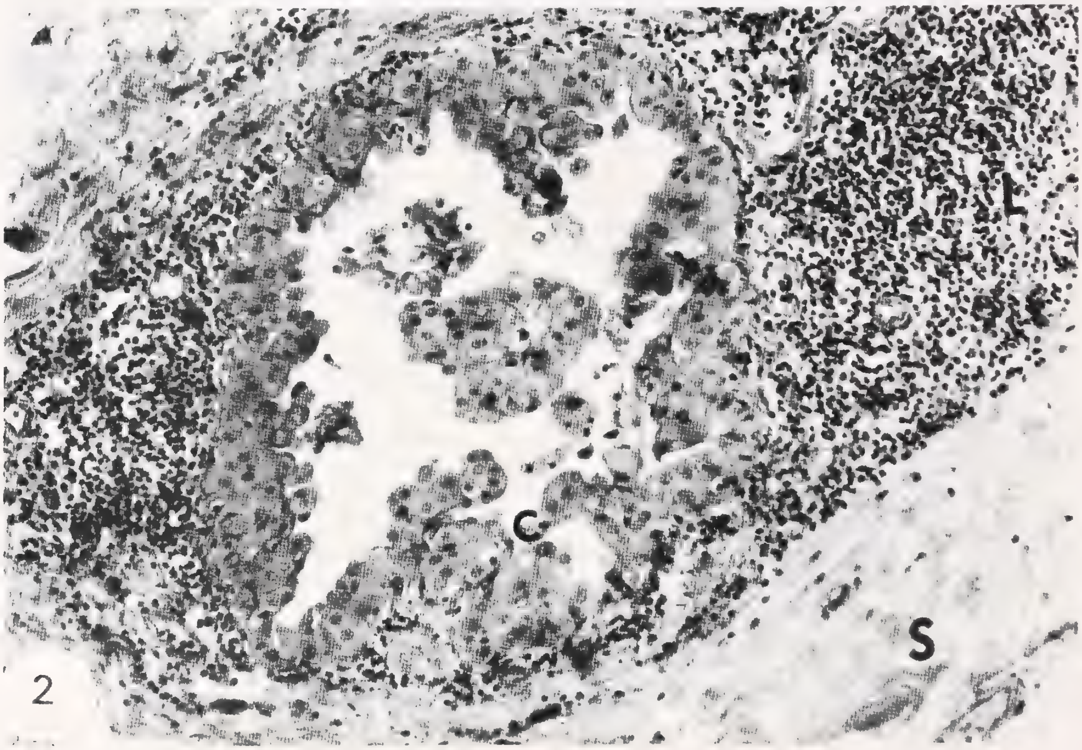
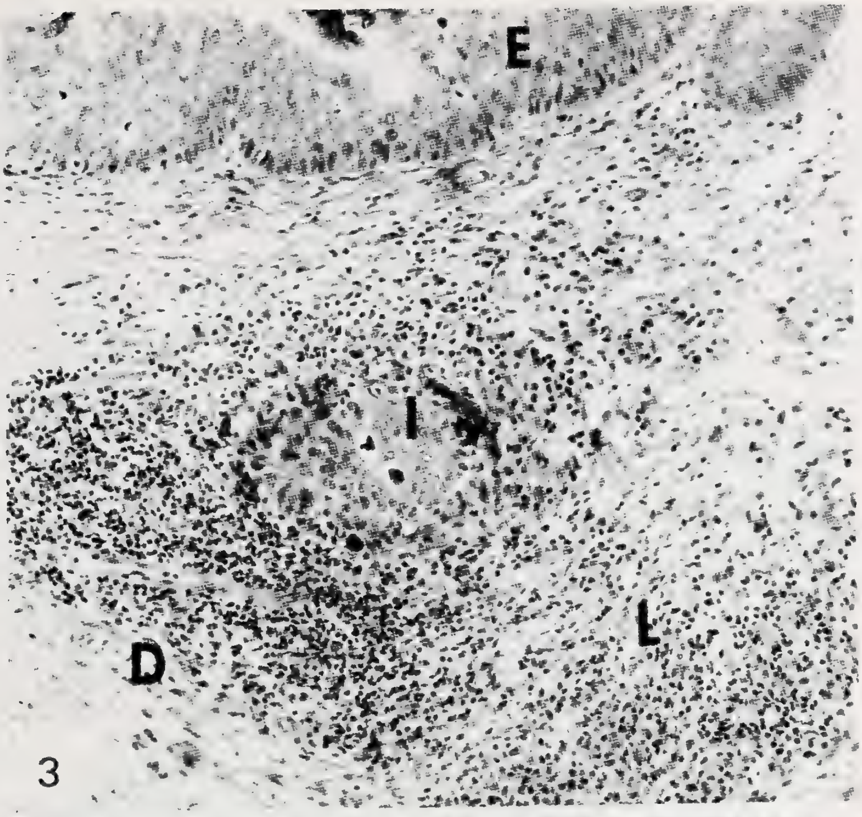


FIGURE 1.—Squamous cell carcinoma of uterine cervix. Cord of tumor cells (T) invading the myometrium (M) is surrounded by abundant infiltrate of lymphocytes (L). HPS. × 100

FIGURE 2.—Intraductal carcinoma in situ. Mammary duct is lined by multiple layers of carcinoma cells (C) that do not invade surrounding fibroconnective stroma (S). Heavy lymphocytic infiltrate selectivity surrounds neoplastic duct. HPS. × 100



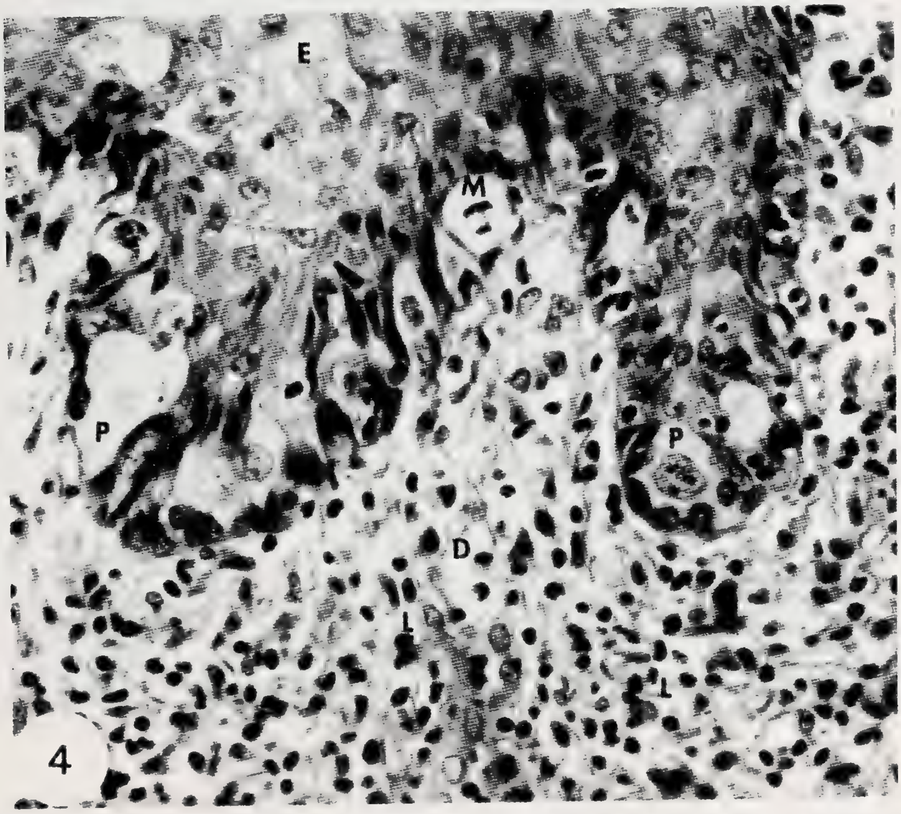
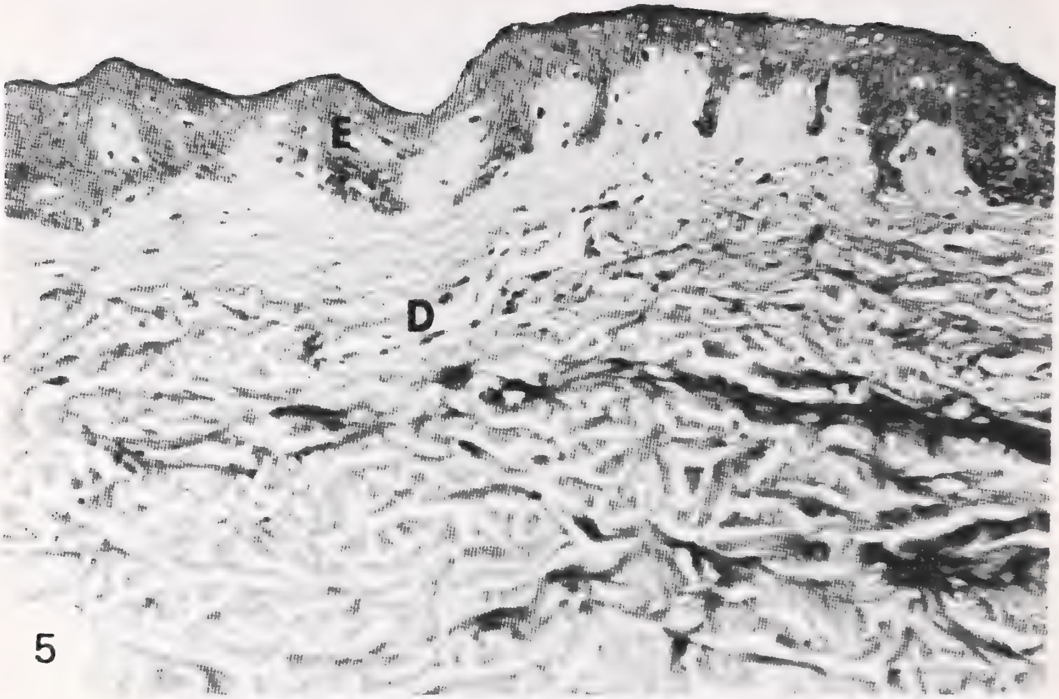


FIGURE 3.—Squamous cell carcinoma in situ of epidermis (E) (Bowen's disease), with focus of invasive squamous cell carcinoma (I) in dermis (D). *Note* abundant lymphoplasmacytic infiltrate (L) in area of tumor invasion. HPS. × 100

FIGURE 4.—Paget's disease of nipple. Epidermis (E) includes multiple carcinoma (Paget) cells (P). Although tumor cells do not invade subjacent dermis (D) (carcinoma in situ), dermis is heavily infiltrated by lymphocytes (L). *Note* tumor cell in mitosis (M). HPS. × 250



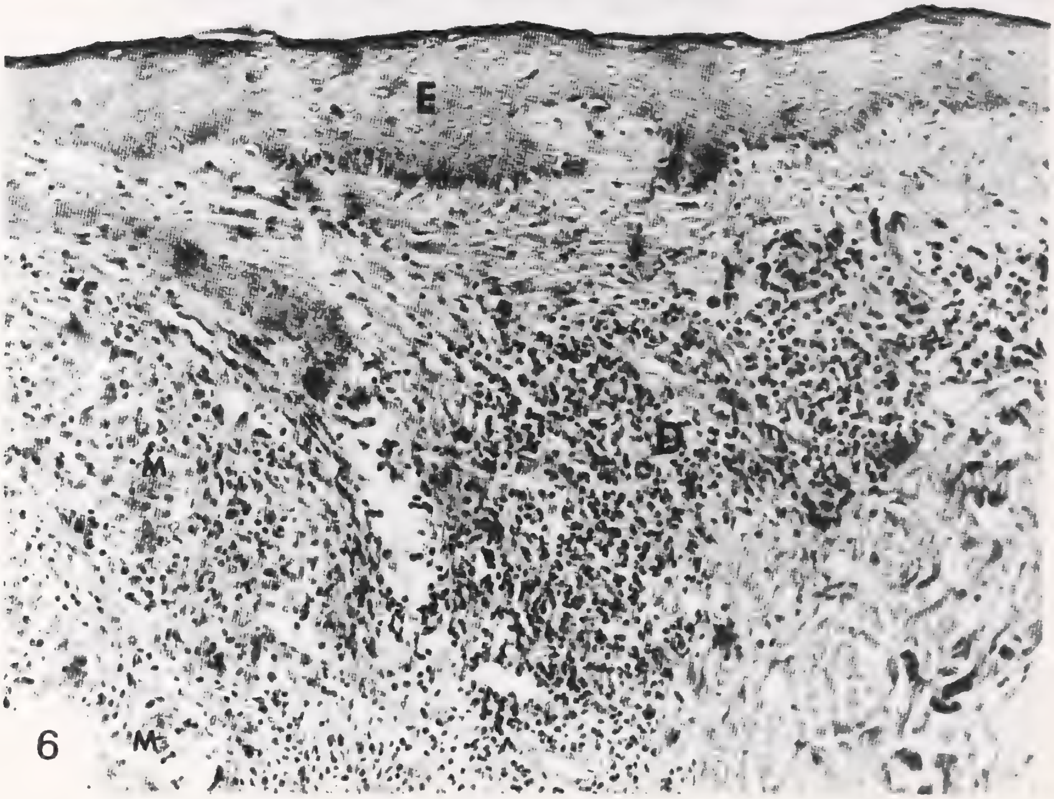
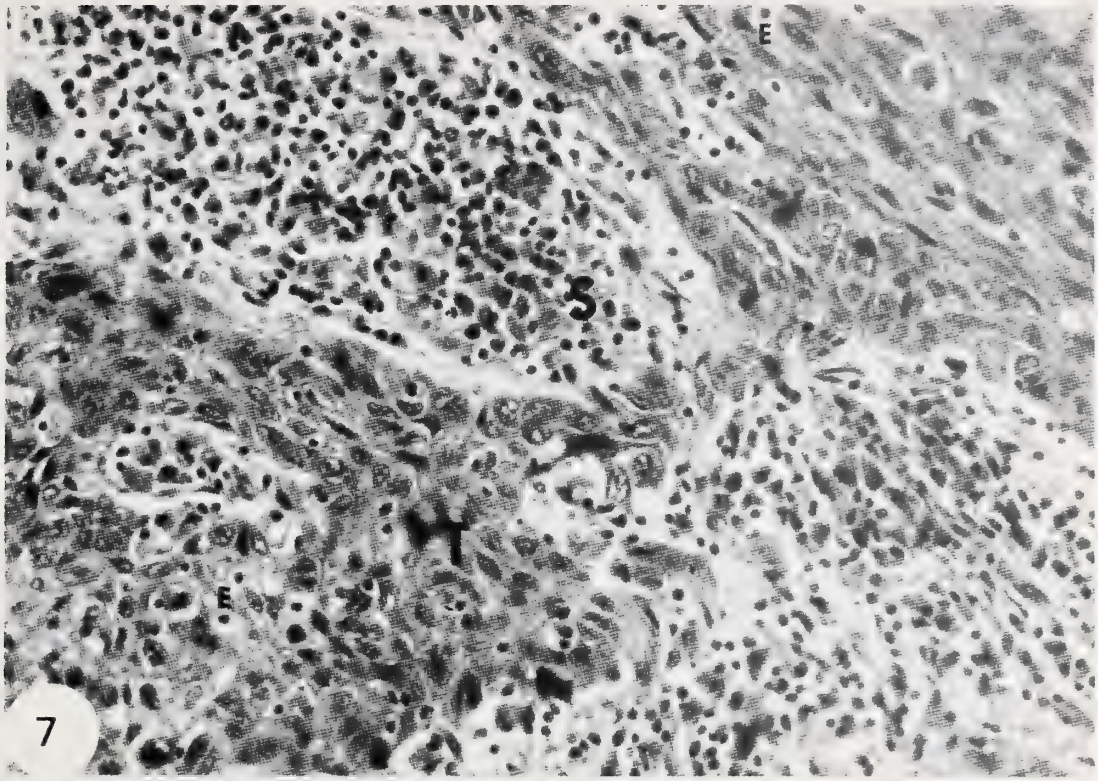


FIGURE 5.—Normal epidermis (E) and dermis (D) at periphery of melanoma (fig. 6). No cellular infiltration is evident. HPS. × 100

FIGURE 6.—Continuation of epidermis (E) and dermis (D) in figure 5. Dermis is heavily infiltrated with lymphocytes and plasma cells in response to presence of small clusters of melanoma cells (M). HPS. × 100



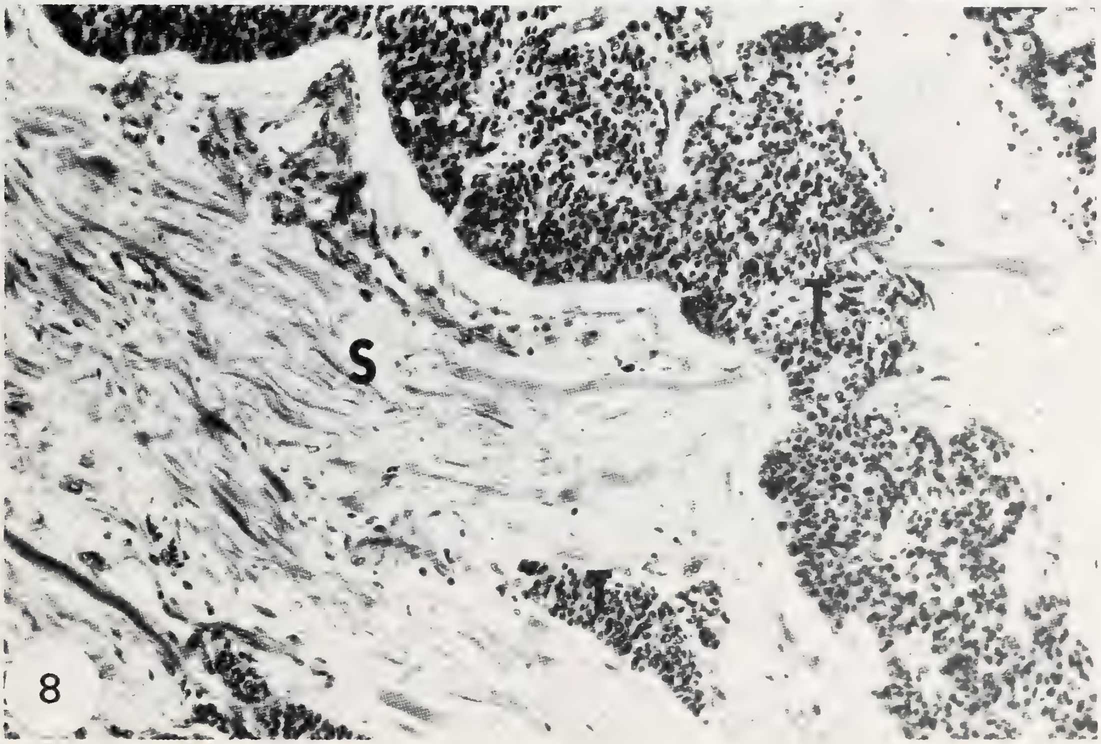
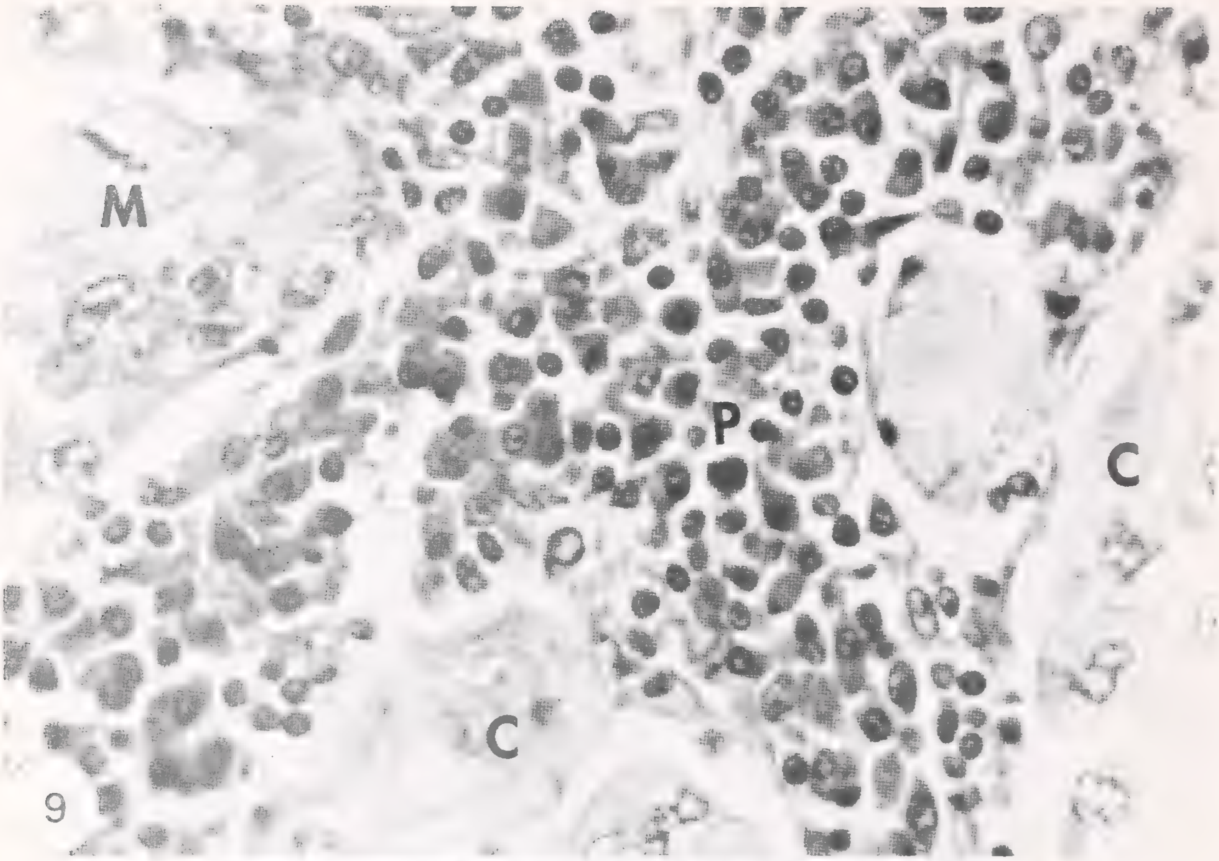


FIGURE 7.—Squamous cell carcinoma of lung. Broad trabeculae of tumor tissue (T), separated and surrounded by masses of cell infiltrates (S), are composed mainly of plasma cells. Numerous lymphocytes have penetrated in and between tumor cells [emperipolesis (E)]. HPS. × 100

FIGURE 8.—Oat cell carcinoma of lung. Trabeculae of poorly differentiated tumor cells (T) are separated by bands of fibrocollagenous stroma (S) with no cellular response. HPS. × 100



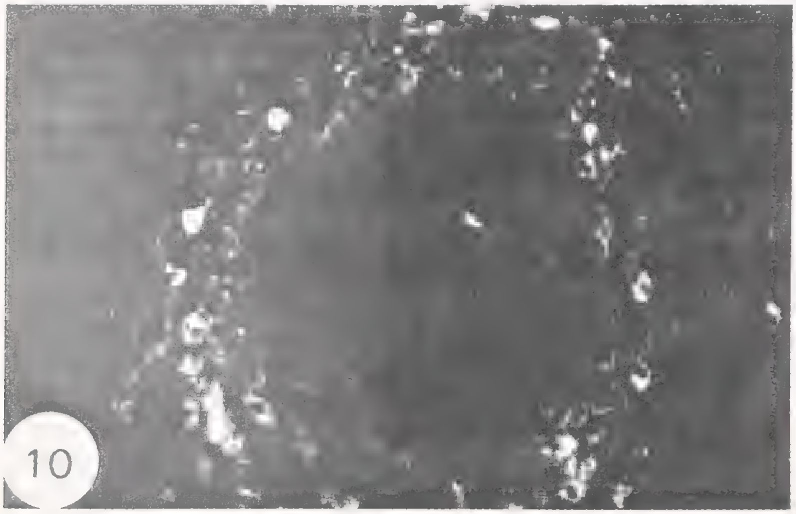


FIGURE 9.—Mediastinal lymph node with metastatic squamous cell carcinoma of lung (C). Lymphocytes in the lymph node have been almost entirely replaced by well-differentiated plasma cells (P). *Note* tumor cell in mitosis (M). HPS. × 250

FIGURE 10.—Squamous cell carcinoma of uterine cervix (same as fig. 1), reacted in direct immunofluorescence with fluorescein isothiocyanate-labeled antiserum to human IgG. Plasma cells and lymphocytes stain positively. × 250

GUEST EDITORIAL

by E. W. Lamon [1, 2, 3]

JNCI 59:769–774, 1977

Stimulation of Tumor Cell Growth In Vitro: A Critical Evaluation of Immunologic Specificity [4]

> *"There is one great difficulty with a good hypothesis. When it is completed and rounded, the corners smooth and the content cohesive and coherent, it is likely to become a thing in itself, a work of art. It is then like a finished sonnet or a painting completed. One hates to disturb it. Even if subsequent information should shoot a hole in it, one hates to tear it down because it once was beautiful and whole."*
>
> —*John Steinbeck,* Log from the Sea of Cortez

During the past decade, two major hypotheses have emerged to explain progressive tumor growth in the face of an immune response; both proposed that a component or components of the immune response promote the growth of the tumor or protect the tumor to the detriment of the host. Both had in vitro correlates, which demonstrated increased tumor cell survival in the presence of components of the immune system, including either immune lymphocytes alone or immune lymphocytes combined with immune serum.

The first of these hypotheses emerged from the immunologic enhancement of allografted tissues (*1*). The observation that tumor cells transplanted into an allogeneic recipient were enhanced in their growth when administered with immune serum (*1*) was extended to a number of syngeneic tumor systems [(*2, 3*), for review]. The in vitro correlate of this phenomenon was proposed to be the blocking of cell-mediated cytotoxicity by immune serum from tumor-bearing hosts (*2, 3*). The blocking factors were thought to be antibodies with specificity for the tumor target cells, which would cover up antigenic sites and thus prevent attachment and subsequent tumor cell lysis by the immune lymphocytes (*2*). This was later modified to include antigen-antibody complexes (*3, 4*) and tumor antigen (*3, 5*) as blocking factors.

The second hypothesis, that of immunostimulation, proposed that tumor-specific immunity at levels inadequate for the production of tumor regression instead produced stimulation of tumor growth (*6*). Subsequent experimentation showed that chemically induced tumor cells, inoculated into thymectomized, irradiated, syngeneic recipients, grew slightly better if administered with immune spleen cells than with normal spleen cells, when both were given at low concentrations (*7*). The in vitro correlate for immunostimulation was proposed to be the specific stimulation of tumor cell growth by small numbers of tumor-immune lymphocytes (*8, 9*).

To reiterate briefly, the common denominator of both hypotheses is that specific immune responses against tumor-associated antigens specifically enhance tumor cell growth.

During my investigations of the immune responses to tumor-associated antigens, I have observed the stimulation of tumor cell growth in vitro, including stimulation by lymphocytes alone and stimulation in the presence of serum and lymphocytes combined. During these studies, a number of specificity controls for lymphocyte cytotoxicity, as well as for antibody-dependent cell-mediated cytotoxicity, were established.

The following is a critical evaluation of the immunologic specificity of the observed stimulation of tumor cell growth; in it, I have used the same rigorous specificity criteria that have been applied to tumor cell cytotoxicity or tumor cell reduction in vitro. The data clearly indicate that, whereas cytotoxicity was demonstrated to be immunologically specific by the criteria used, the stimulation of tumor cell growth in vitro was not specific and, furthermore, may not be immunologic in nature.

CRITERIA FOR SPECIFICITY

Lymphocyte Activity

1) The activity of the immune lymphocytes should be compared to the activity of control lymphocytes from nonimmune animals. When fractionated lymphocytes are utilized, the activities of the immune fractions should be compared with the activities of the same fraction of control

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ABBREVIATIONS USED: M-MuSV = Moloney strain of murine sarcoma virus; SRBC = sheep red blood cell(s); SV40 = simian virus 40; H-MuSv = Harvey strain of murine sarcoma virus; M-MuLV = Moloney strain of murine leukemia virus.

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

lymphocytes. 2) The activity of immune lymphocytes should be compared to the activity of control lymphocytes that are immune to a nonpertinent antigen. 3) The immune lymphocytes should be tested on control target cells that do not possess the antigen in question. 4) Reciprocal specificity controls should be performed with an antigenically distinct tumor to show that the effect is producible only with the appropriately immune lymphocytes on the pertinent target cells.

Combined Serum and Lymphocyte Activity

1) The activity of the immune serum in combination with lymphocytes should be significantly different than the activity of the lymphocytes alone. 2) The effect should not be demonstrable with combinations of immune serum and lymphocytes on a control target cell. 3) The effect should not be produced by the serum or fraction thereof, without lymphocytes, on the pertinent target cell. 4) The effect should not be produced by serum from control animals and lymphocytes. 5) Reciprocal specificity controls should be performed with lymphocytes and immune serum with specific activity against another tumor target cell.

OBSERVATIONS

Activity of Unfractionated Lymphocytes

Using the microcytotoxicity assay (*10*) to evaluate the cytotoxic lymphocyte response to primary tumors induced by M-MuSV, we found that specific cytotoxic lymphocytes were present soon after the injection of virus, prior to tumor development, and following the regression of tumors (*11, 12*). However, lymphocytes obtained from animals at peak tumor size (approximately 15 days after virus injection) consistently were hyporeactive against the specific target cells (*11–14*). In many instances, these lymphocytes stimulated target cell growth significantly, compared to the growth of target cells in wells with control lymphocytes from uninjected animals (*11, 12, 14*). However, spleen cells from M-MuSV-infected animals also stimulated control (D56) target cell growth (table 1, group I). D56 cells contain a defective sarcoma virus genome and do not possess a surface antigen that distinguishes them from untransformed cells (*15*). Thus even though lymphocytes from some animals infected with M-MuSV stimulate growth of the pertinent target cell (Ha2), such lymphocytes also stimulate the growth of control target cells. Therefore, by this criterion the stimulation of target cell growth is not immunologically specific.

Also, lymphocytes from animals immune to SRBC, allogeneic spleen cells, or an allogeneic chemically induced tumor (MLB) (*16*) stimulated Ha2 target cell growth (table 1, group II. Thus immunization against nonpertinent antigens produced stimulation of target cell growth.

In another series of experiments, we immunized mice against polyoma virus-transformed cells and found that lymphocytes from some of these animals stimulated target cell growth. This occurred particularly when the final immunizing dose of polyoma-transformed cells was suboptimal for the production of specific cytotoxicity (*17*). Lymphocytes from animals inoculated with polyoma-transformed cells stimulated the growth of not only polyoma-transformed cells but also SV40-transformed cells, Ha2 cells, normal 3T3 cells, and primary embryo fibroblasts (table 1, group III). These specificity controls with the polyoma system indicated that the stimulation of target cell growth with unfractionated lymphocytes was not immunologically specific. Also, the greatest magnitude of stimulation of target cell growth was not always produced by the lowest numbers of lymphocytes.

Activity of Fractionated Lymphocyte Subpopulations

In the M-MuSV system, specific lymphocyte cytotoxicity in long-term regressor animals (30 days and later after injection of the virus) was dependent on the presence of immune

TABLE 1.—*Stimulation of target cell growth by unfractionated lymphocytes*

Group[a]	Lymphocvtes[b]		Target cells[c]			Percent of control[d]	Lymphocyte concentration[e]
	Donor	Immune to	Designation	Strain of origin	Viral transformant		
I	BALB/c	M-MuSV	D56	NIH Swiss	M-MuSV-defective	149±15	High
II	BALB/c	SRBC	Ha2	CBA	H-MuSV-M-MuLV	142[f]	Low
II	CBA	C57BL spleen	Ha2	CBA	H-MuSV-M-MuLV	128	Low
II	$(A.CA \times CBA)F_1$	MLB	Ha2	CBA	H-MuSV-M-MuLV	140	High
III	C3H	Py4198	Py4198	C3H	Polyoma	142	Low
III	C3H	Py4198	SVT	BALB/c	SV40	201	High
III	C3H	Py4198	Ha2	CBA	H-MuSV-M-MuLV	180	High
III	C3H	Py4198	Embryo fibroblasts	C3H	Untransformed	241	Low
III	C3H	Py4198	3T3	BALB/c	Untransformed	234	Low

[a] Group I: Data were taken from (*9*); group II: unpublished observations; group III: Walia AS, Lamon EW: Manuscript in preparation.

[b] Group II immunization was described in (*12*) for SRBC and in (*16*) for MLB and C57BL spleen cells. Immunization schedule for group III was described in (*17*).

[c] Concentrations/well: groups I and II, 50–100; group III, 200. Group I and II assays were done for 48 hr; group III, for 24 hr.

[d] Calculated as in (*11*). The numbers shown are the maximum stimulation for each expt. The value for group I represents the maximum stimulation ± SD from 4 expts.

[e] Lymphocyte concentration producing maximum stimulation. "High" indicates >5,000; lymphocytes/10-μl well; "low" indicates ≤5,000 lymphocytes/well.

[f] Compared to blank control.

B-cells (*18, 19*). Removal of B-lymphocytes from the immune spleen cell populations eliminated the cytotoxic activity against the pertinent (Ha2) target cells. Often these T-cell-enriched populations stimulated Ha2 target cell growth compared to lymphocytes from control animals fractionated in an identical manner (*19*). However, this stimulation was also observed with the control D56 target cells (table 2, group I). Furthermore, T-lymphocytes from animals immune to nonpertinent antigens (in these cases, SRBC or an alloantigen) also stimulated the growth of the Ha2 target cells (table 2, group II). In some experiments, Ha2 target cell growth was stimulated by lymphocytes that had been depleted of T-cells (*13*). This, however, was not specific for the Ha2 target cells. The control D56 target cells were also stimulated by immune B-cells (table 2, group III).

According to the criteria for specificity as outlined, stimulation of tumor target cell growth by either unfractionated immune lymphocytes or fractionated subpopulations of immune lymphocytes under these experimental conditions is not immunologically specific. It is not even tumor associated, inasmuch as we have observed a stimulation of untransformed 3T3 cells and primary embryo fibroblasts by lymphocytes from animals inoculated with polyoma tumor cells.

Combined Activities of Lymphocytes and Serum

During our early studies of the combined activities of immune serum and lymphocytes in the M-MuSV system, we found that immune sera had two apparent effects on the cytotoxicity of immune lymphocytes. At relatively high serum concentrations, the cytotoxicity of the immune lymphocytes was potentiated (*19*). However, at lower serum concentrations, the potentiation effect was lost and with some sera a "blocking" effect was found (*20*). In some cases, this went much beyond a simple blocking of cell-mediated cytotoxicity, and stimulation of target cell growth was observed (*20*). Following the specificity criteria evolved for use in evaluating antibody-dependent cell-mediated cytotoxicity, one can assess whether this blocking of cytotoxicity or stimulation of target cell growth in the presence of immune serum and lymphocytes can be determined to be immunologically specific.

TABLE 2.—*Stimulation of target cell growth by fractionated lymphocytes*

Group^{a}	Lymphocytes^{b}			Target cells^{c}	Percent of control^{d}
	Donor	Immune to	Fraction		
I	BALB/c	M-MuSV	T-cells	Ha2	152±30
				D56	135±15
II	BALB/c	SRBC	T-cells	Ha2	193^{e}
II	CBA	C57BL spleen	T-cells	Ha2	143
III	BALB/c	M-MuSV–M-MuLV	B-cells	D56	176±18

[a] Group I: T-cell-enriched populations; data were from (*18, 20*). Group II: T-cell-enriched populations (unpublished observations). Group III: B-cell-enriched populations; data from (*12, 18, 20*).

[b] Immunization of group II was as described in (*12*) for SRBC and (*16*) for C57BL spleen cells.

[c] Initial target cell concentration was 100–200 cells/well; incubation time was 24 hr.

[d] Mean±SD of the maximum stimulation from either 6 expts (group I) or expts using two different fractionation techniques for T- and B-cell enrichment (group III). Values for group II represent maximum stimulation from single expts, the mean of 6 replicates each. Differences between stimulation of Ha2 and D56 are not significant.

[e] Compared to blank control.

Activity of Unfractionated Serum

In recent investigations of the combined activity of antibody and lymphocytes in the M-MuSV system, we evaluated autochthonous combinations of serum and spleen lymphocytes from animals that had been infected by M-MuSV (*14*). Sera from regressor mice potentiated the cytotoxic activity of autochthonous lymphocytes. Also, sera from tumor-bearing animals at 15 days after M-MuSV injection induced cytotoxicity by autochthonous lymphocytes that were not by themselves cytotoxic. We did, however, find some instances of stimulation of target cell growth in this study. Serum from uninfected animals in combination with regressor lymphocytes produced significant stimulation of Ha2 target cell growth (table 3, group I). This effect has also been observed with combinations of normal serum and normal lymphocytes (*21*). Also, immune serum from a regressor animal in the absence of lymphocytes stimulated Ha2 target cell growth in comparison to that of the blank control (table 3, group I).

In testing antibody-dependent cell-mediated cytotoxicity in the M-MuSV system, we found that immune serum from M-MuSV regressor animals induces specific cytotoxicity by thymocytes as well as lymph node and spleen cells (*22–25*). Unfractionated regressor serum on control target cells in combination with lymphocytes from all these organs, however, produced stimulation of growth (table 3, group II).

TABLE 3.—*Stimulation of target cell growth by unfractionated serum*

Group^{a}	Lymphocytes^{b}		Serum^{c} specificity	Target cells^{d}	Percent of control^{e}
	Donor	Immune to			
I	BALB/c	M-MuSV	Control	HA2	142
I	None	—	M-MuSV–M-MuLV	Ha2	179^{f}
II	CBA	Control	M-MuSV–M-MuLV	Py3T3	141
				D56	179
III	C3H	Control	Polyoma	Ha2	138
				SVA	147
IV	CBA	Control	M-MuLV	3T3	482

[a] For group I, data were taken from (*14*); for group II, from unpublished observations; for group III, from Walia AS, Lamon EW: Unpublished observations; for group IV, from (*25*).

[b] Concentrations: 10,000 cells/well (groups I, II, and III); 5,000/well (group IV).

[c] For groups I and II, sera were from M-MuSV regressor animals; for group III, from animals hyperimmunized with polyoma-transformed cells (*16*). The immunization schedule for group IV is detailed in (*25*).

[d] Initial concentrations: 200 cells/well (groups I, II, and III); 50/well (group IV). Incubation time was 24 hr for groups I, II, and III; 48 hr, for group IV.

[e] Calculated as in (*19*). Numbers shown are the maximum stimulation for each expt. All numbers shown are significantly ($P<0.05$) different than the control and were produced by serum dilutions of 1:80. Groups II and III represent mean maximum stimulation with spleen, lymph node, and thymus cells incubated with the serum and target cells indicated.

[f] Serum alone compared to blank control.

In experiments with antiserum immune to polyoma-transformed cells, we found that the polyoma-immune serum was also capable of inducing antibody-dependent cell-mediated cytotoxicity against polyoma-transformed cells by spleen, lymph node, and thymus cells (Walia AS, Lamon EW: Unpublished observations). However, the same combinations of serum and lymphocytes stimulated the growth of Ha2 and SVA target cells (table 3, group III). Thus serum immune to a nonpertinent antigen in combination with lymphocytes stimulated target cell growth.

In a series of experiments evaluating the immune response to allogeneic Moloney lymphoma cells in mice, we tested two groups of sera for antibody capable of inducing cell-mediated cytotoxicity. One group contained only viral-specific antibody and another group contained both viral-specific and alloantigen (H-2^d)-specific antibodies (*26*). Testing of these sera on BALB/3T3 target cells for antibody-dependent cell-mediated cytotoxicity revealed that the serum containing alloantibody produced significant cytotoxicity by normal spleen cells (*26*). However, the antiserum that contained no detectable alloantibody activity against BALB/3T3 cells produced stimulation of 3T3 cell growth in the presence of lymphocytes (table 3, group IV). This serum did contain antibody activity specific for antigens determined by M-MuLV (*26*). However, BALB/3T3 cells do not possess any M-MuLV-determined antigens. Thus antiserum immune to M-MuLV antigens in combination with lymphocytes produced stimulation of control target cell growth (in this case, untransformed 3T3 cells). The same serum and lymphocyte combination that produced this stimulation of 3T3 cell growth was cytotoxic against Ha2, an M-MuLV antigen-positive cell line.

Activity of Fractionated Serum

IgG and IgM from M-MuSV regressor animals induce cytotoxicity by normal lymphocytes and potentiate the cytotoxic activity of immune lymphocytes against Ha2 target cells (*21*). In some experiments, Ha2 target cell growth was stimulated at lower immunoglobulin concentrations (*21*). However, testing of IgM and IgG from regressor animals also produced a stimulation of control target cell growth (table 4, group I). Thus the antiserum fractions in combination with lymphocytes stimulated control target cell growth. Finally, in one set of experiments, we selectively removed IgG and/or IgM from M-MuSV regressor serum by passing it over specific immunoadsorbent columns. As a control for the efficacy of each column, we also passed the serum sequentially over both columns to test for remaining activity (*22*). Although all of the activity in terms of antibody-dependent cell-mediated cytotoxicity was removed by this procedure, the IgG- and IgM-depleted effluent serum in combination with lymphocytes stimulated Ha2 target cell growth (table 4, group II).

TABLE 4.—*Stimulation of target cell growth by fractionated serum*

Group[a]	Donors of Lymphocytes[b]	Sera[c]		Target Cells[d]	Percent of control[e]
		Immune to	Fraction		
I	CBA	M-MuSV-M-MuLV	IgG	Py3T3	140
I	CBA	M-MuSV-M-MuLV	IgM	Py3T3	133
II	BALB/c	M-MuSV-M-MuLV	IgM & IgG removed	Ha2	146

[a] For group I; data were from (*24*); for group II, cytotoxicity data were from (*21*).

[b] Concentration: 10,000/well.

[c] From M-MuSV regressor animals. The IgG and IgM fractions in group I were obtained by Sephadex G-200 fractionation. Immunoadsorbent columns used in Group II are described in (*21*).

[d] Initial concentration: 200/well. Incubation time: 24 hr.

[e] Calculated as in table 3. Only maximum values are shown; these were produced by dilutions of ≤1:40.

Serum factors, in combination with lymphocytes and in some instances in the absence of lymphocytes, can nonspecifically promote tumor cell growth in vitro. Serum plus lymphocytes often produced a stimulation of target cell growth that was significantly greater than the growth of target cells with lymphocytes alone. However, control target cells and untransformed 3T3 cells were also stimulated to grow by combinations of lymphocytes and sera, including the IgG and IgM fractions of antisera. Immune serum alone produced a significantly increased cell growth compared to the blank control, and in some instances, control serum and immune lymphocytes produced stimulation of target cell growth. Reciprocal specificity controls revealed that target cell growth was stimulated by serum immune to a nonpertinent antigen in combination with lymphocytes. Finally, removal of IgM and IgG from immune serum and testing of the immunoglobulin-depleted serum in combination with lymphocytes stimulated target cell growth.

We have, in many cases, illustrated stimulation of target cell growth by control lymphocytes in combination with sera. However, the magnitude of the stimulation shown would be adequate to obliterate a cytotoxic effect by immune lymphocytes. For example, if immune lymphocytes were capable of producing a 30% target cell reduction, a 30% nonspecific stimulation of target cell growth would result in an apparent 100% blocking. As shown in the tables, the stimulation was often of this magnitude or greater.

DISCUSSION

The interpretation that the observed target cell growth stimulation is not immunologically specific is dependent partly on a lack of cross-reactivity of the control target cells and the pertinent target cells. One could argue, for example, that the D56 cells used as control target cells in the M-MuSV experiments possess weak surface antigen(s) determined by the defective sarcoma virus. This is especially pertinent in light of the findings by some investigators that M-MuSV-transformed nonproducer cells containing a defective sarcoma genome possess serologically detectable antigens (*27*). Such a weak antigenicity might promote target cell growth in the presence of antibodies or immune lymphocytes. The following evidence, however, would seem to make this argument untenable. Extensive serologic studies with antisera from M-MuSV-immune animals or appropriately absorbed alloantisera have revealed no surface antigen that would distinguish the D56 cells from their untransformed 3T3 cell counterparts (*15*). Also, studies with lymphocytes from M-MuSV-infected hosts show the specificity of the lymphocyte cytotoxicity to be identical with serologic reactivity (*11, 12, 15, 17, 18, 20*). Other investigators using isotope release assays have not found the lymphocyte cytotoxicity to parallel the serologic specificities in the M-MuSV system (*28*) as we have in our studies. However, using a sensitive competitive assay these same investigators have found that the D56 cells possess no antigens that can competitively inhibit the lysis of relevant target cells by M-MuSV-immune lymphocytes (*28*). This confirms our contention that D56 cells have no antigens recognizable by lymphocytes from M-MuSV-immune hosts.

Using reciprocal specificity controls with the polyoma system, we have found that cytotoxic lymphocytes and antibody-dependent cell-mediated cytotoxicity are specific for target cells possessing the relevant antigen(s). Antibodies and lymphocytes from M-MuSV regressor animals are cytotoxic against Ha2 target cells possessing M-MuLV-determined cell-surface antigen(s) but are not cytotoxic against polyoma-transformed cells (*25*). Immune lymphocytes alone or lymphocytes plus antibodies from animals immunized against polyoma-transformed cells are cytotoxic against polyoma-transformed cells in vitro but not against the Ha2 cells possessing M-MuLV-associated cell-surface antigens. This pattern of specificity is not demonstrable in terms of target cell growth stimulation. Indeed, just the opposite is found. When antisera and/or lymphocytes are tested on target cells that lack the relevant antigen(s), the target cells grow better.

A further argument could be made that all of the target cells used have common antigens (for example, embryonic antigens) and that weak immunologic reactivity against such antigens is responsible for stimulating target cell growth. This would not explain, however, the increase in target cell growth produced by immunization against SRBC or allogeneic spleen cells (*see* tables 1, 2). Furthermore, the increases in target cell growth produced by the combination of normal serum and lymphocytes or by immunoglobulin-depleted serum in combination with lymphocytes, could not be accounted for by immunologic specificity against cross-reactive antigens.

One must also consider the influence of natural antibodies and natural cytotoxic effector cells [reviewed in (*29*)]. Conceivably the stimulation of target cell growth we have observed by immune lymphocytes could be an absence of natural cytotoxicity in the antigenically stimulated population as compared to control lymphocytes from unimmunized hosts. Also, the possibility of natural antibodies blocking natural cell-mediated cytotoxicity should be considered in the evaluation of target cell growth stimulation by lymphocytes and serum combined. However, a careful analysis of the data indicates that these two situations cannot account for the spectrum of target cell growth stimulation observed. A blocking of natural cell-mediated cytotoxicity by natural antibodies would not explain the stimulation of target cell growth by immunoglobulin-depleted serum or the stimulation by serum alone. Furthermore, in many instances, the target cell growth observed was significantly greater than that in blank controls in the same experiment, which contained only target cells and medium (*see* tables 1, 2). Thus growth stimulation by immune lymphocytes in these cases cannot be considered only as a lack of natural cytotoxicity if one accepts the medium control without lymphocytes as being the base line for no cytotoxicity.

If stimulation of target cell growth in vitro, under these experimental conditions, is not immunologically specific, we are left with two general possibilities. First, the phenomenon could be immunologic in nature, yet not specific. A number of well-documented immunologic phenomena are nonspecific: e.g., the cytotoxicity of lymphocytes activated by mitogens (*30*), the activity of supressor T-cells in many instances (*31*), and the activity of some lymphokines (*32*). Conceivably, stimulation of target cell growth in vitro by immune elements could lack specificity as defined above and yet be immunologic in nature. Some substances possibly produced by lymphocytes and combinations of lymphocytes and antiserum may nonspecifically stimulate adherent target cell growth in vitro. Small doses of mouse lymphotoxin have just such an effect (*33*). The second possibility may be more trivial (from an immunologic point of view). The growth of cells in tissue culture can be promoted by the addition of nutrients (*34*), e.g., serum (*35*). Furthermore, some cell lines have their growth enhanced by "conditioned" medium (*36*). Thus immune lymphocytes or the combination of serum and lymphocytes may provide an environment that is nutritionally more advantageous to the target cells. In this case, the phenomenon would fall into the realm of nutritional physiology and not immunology.

Our in vitro findings with regard to stimulation of target cell growth may or may not have relevance to the in vivo situation of tumor enhancement, but hopefully these observations will emphasize the need for rigorous specificity controls for both in vitro and in vivo experiments.

CONCLUSIONS

Many situations stimulate tumor target cell growth in vitro, e.g., immune lymphocytes and various combinations of serum and lymphocytes. Whereas the target cell reduction or cytotoxicity by immune lymphocytes or immune serum and lymphocytes combined is immunologically specific according to rigorous specificity criteria, the stimulation of target cell growth, by the same criteria, is not specific. Furthermore, the stimulation of target cell growth in vitro cannot even be tumor associated, since untransformed cells are also stimulated by immune lymphocytes and combinations of serum and lymphocytes.

The evidence presented here serves little useful purpose if it only detracts from one hypothesis or group of hypotheses without suggesting an alternative or providing support for another. The alternative suggested, admittedly not new, is that specific immune responses against tumor-associated antigens are detrimental to tumor cells. We (and many others) have documented cytotoxicity by tumor-immune lymphocytes and antibody-dependent cell-mediated cytotoxicity with antibodies and lymphocytes, and these phenomena fulfill the rigorous specificity criteria described. Considering the factors that can produce nonspecific stimulation of target cell growth, our estimates of cytotoxicity are probably minimal. Thus the specifically immune lymphocytes or combinations of antibodies and lymphocytes must first overcome the nonspecific influences that stimulate tumor target cell growth before significant levels of cytotoxicity can be observed.

The proposal that specific immune responses against tumor-associated antigens are detrimental to tumor cells does not imply that tumors cannot escape immunosurveillance and grow; they do. Many "escape" mechanisms exist that do not require a specific stimulation of tumor growth by the immune response against it (*37-41*).

These factors considered, the specific immune response to tumor-associated antigens as a tumoricidal or tumor inhibitory phenomenon provides a hypothetical framework supported by a large body of in vitro and in vivo evidence. Within this framework is the possibility of using the immune response to achieve successful tumor prevention and specific tumor immunotherapy.

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GUEST EDITORIAL——by **Richmond T. Prehn**, *M.D.* [1, 2]

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Immunostimulation of the Lymphodependent Phase of Neoplastic Growth

Most oncologists are probably of the opinion that the immune system constitutes an important defense against the occurrence of cancer, a concept embodied in the term "immunologic surveillance" (*1*). However, increasing numbers of cognoscenti are now persuaded that the role of immunity has, in most tumor systems, been vastly overrated (*2–5*). I have begun to entertain the diametrically opposite hypothesis that an immune reaction, rather than killing incipient neoplasms, may actually stimulate them to grow (*5–7*). My purpose in this editorial is not to persuade you that the immune reaction is a significant cause of tumor growth, but rather to persuade you that this hypothesis merits serious consideration.

Before one questions whether the action of an immune response is predominantly stimulatory or inhibitory to target tumors, it would be well to ask whether or not the neoplasm stimulates an immune response of any kind. The answer may be affirmative in some systems, but it is by no means clear that this is always so, or indeed that this is frequently so.

There appears to be no question about the fact that most tumors induced in the laboratory, either by viral, chemical, or physical agents are, at least potentially, immunogenic (*8*). The growths of transplants of these tumors can often be modified by specific immunization; in vitro tests of various kinds are often positive, and immunodepression of the host animals often leads to faster growth of transplants. Furthermore, in some viral systems it is clear that immunodepression potentiates the appearance and growth of the primary, untransplanted neoplasm, and conversely, that vaccination is successful (*8*).

In chemical tumor induction, potentiation of oncogenesis by immunodepression is difficult to demonstrate, perhaps partly because of the immunodepression produced by the oncogen itself (*9*). Positive, statistically significant reports exist, but they are nonetheless usually of marginal degree (*10, 11*). Failures have also been revealed, and one suspects that many negative results have not been reported (*2*). Under these circumstances, the real significance of the positive reports remains questionable.

Most important is the fact that so-called spontaneous tumors in rodents, i.e., tumors that arise in low frequency, often in older animals and for no overt cause, are seldom immunogenic (*12, 13*). (By immunogenic, I refer only to the capacity of a tumor to induce an immunity in vivo that will restrict to some degree the subsequent growth of implants.) In chemically induced tumors, immunogenicity tends to be directly related to the concentration of the inducing oncogen; low concentrations that presumably mimic the spontaneous condition produce tumors of little or no immunogenicity (*14*). The reason for the low or nonexistent immunogenicity of spontaneous tumors or those induced by low doses of carcinogen is not yet known, and indeed it may vary from case to case. An absolute paucity of tumor-associated antigens is to be suspected, but alternatives such as induction of "blocking" factors, shedding of antigen, elicitation of suppressor cells, or induction of a competing tumor-stimulatory reaction have, in most instances, not been ruled out.

The possibility of course exists that "spontaneous" tumors might be more immunogenic at their inception and that this property is lost via immunoselection during tumor "progression" (*8*). That some selective effects do occur in relation to tumor immunogenicity has been directly demonstrated by Bartlett (*15*) and Bubeník et al. (*16*). However, tumors that arise in the immunologically free confines of tissue culture or in diffusion chambers are, unless a chemical or virus has been deliberately added, seldom immunogenic. This suggests that immunoselection is not the fundamental reason for the lack of immunogenicity in spontaneous tumors (*15, 17–20*). Immunogenicity seems to be a property initially conferred by the oncogen, if one is overtly present; immunoselection can certainly then occur but need not be invoked to account for the lack of immunogenicity of spontaneous tumors or tumors induced by low doses of carcinogens.

That the immune system does interact with spontaneous human tumors has been forcibly argued by Ioachim (*21*) in defense of the concept of immunologic surveillance. He has rightly pointed out that many human tumors, especially in the early stages of their evolution, are associated with an intense infiltration of lymphocytes and/or related cells. It seems reasonable to suppose that this infiltrate is indicative of immunologic recognition of the neoplasm. Furthermore, the presence of this infiltrate suggests that many human tumors may result from the action of chemical carcinogens and others from the action of oncogenic viruses. How many human neoplasms are really spontaneous in the sense of the spontaneous rodent tumors? In the rodent, the term spontaneous is perhaps best equated with tumors that arise infrequently and sporadically. However, this infrequency is usually in an inbred population in which all the members are at equal genetic risk. In man, where genetically susceptible individuals cannot yet be identified, it is possible that some tumors, thought of as spontaneous, may actually occur in nearly 100% of the genetically susceptible population! Are these then to be considered spontaneous, or are they more akin to those rodent tumors induced in high frequency

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

by a virus or an appreciable dosage of carcinogenic chemical? In the rodent, the chemically induced tumor is the type most typically associated with a lymphoid infiltrate. Thus it is clear that we should be cautious in assuming that spontaneous rodent tumors are a better model of human disease than are those induced by viruses or chemicals.

Apart from the evidence of lymphocytic infiltration, evidence for the immunogenicity of human tumors is, unfortunately, suspect. The numerous in vitro tests designed to demonstrate tumor antigens are, in my estimation, difficult to interpret and their meaning is not yet clear. Human tumor regression, which in some tumor types is frequent, is not necessarily a manifestation of immunity. However, for the sake of argument and the purposes of this editorial, I will take the evidence of lymphoid cell infiltration at face value and assume that many human tumors are indeed recognized as "foreign" by the immune mechanism. We can now consider the possible biologic effects of this recognition.

It is perhaps important to consider at what point in tumor evolution such recognition may occur. As has already been mentioned, the infiltrate of lymphocytic and associated cells sometimes found in human neoplasms is most characteristic of early lesions. The infiltrate is perhaps most dramatic in premalignant skin lesions or in lesions just beginning dermal invasion. In early melanoma of the skin, for example, the pathologist is commonly aided in distinguishing "malignant" from innocuous lesions by the intense lymphocytic dermal infiltration commonly associated with malignant lesions (*22*). At later stages of the disease, the lymphocytic infiltration is usually less intense or may even disappear (*22*).

Although the lymphocytic infiltrate supports the argument that immune recognition may occur early, other data suggest that the lesion must nonetheless reach a size containing hundreds and perhaps thousands of antigenic cells before an immune response can occur. For example, in chemically induced mouse breast cancer, strong evidence exists to indicate that the hyperplastic nodule (the precancerous lesion) usually fails to immunize the host despite the fact that the nodule contains the necessary antigens. Immunization does not occur until the lesion grows and extends outside the breast fat pad (*23*). Likewise, evidence suggests that chemically induced, antigenic, mouse skin tumors may fail to immunize the host when they are left undisturbed in the skin (*24*). These data suggest a lack of antigen recognition in early lesions.

A further argument against the hypothesis that immune recognition occurs when the neoplasm is very small is the phenomenon called "sneaking through." It has been repeatedly observed that an antigenic tumor implant may fail to produce effective immunity against its own growth if the inoculum is sufficiently small (*25-29*). That this curious effect could be the result of any type of immunologic tolerance seems doubtful [although some evidence supports the tolerance hypothesis (*28*)], since the same effect can sometimes be even more pronounced in previously immunized hosts (*29*). The explanation of sneaking through may be that, by the time the lesion is discovered by the immune response, the lesion is too large to be greatly affected by it.

These varied data argue that any effect of immunity on a neoplasm, be it surveillance or stimulation, probably occurs *after* the tumor has reached a respectable size. Even in viral systems, in which immunization may occur prior to, or simultaneously with, neoplastic transformation, the existence of the sneaking through phenomenon in previously immunized animals suggests that the tumor per se would not stimulate a second-set reaction, i.e., attract immunologic attention, while still very small. (However, sneaking through has not, to my knowledge, been sought in a viral tumor system). Thus the original idea of immunologic surveillance, namely, that there is an immune elimination of neoplastically transformed cells or clones virtually as soon as they occur, does not seem tenable. Possibly, mechanisms exist that inhibit or eliminate neoplastically transformed cells or clones virtually at their inception but, if so, these mechanisms do not appear to be immunologic. The fact that the immune mechanism does not interact with the tumor directly until the lesion is of considerable size is important to my overall thesis, as will soon be apparent.

It would be hard to argue that a lymphoid infiltrate, when present, is irrelevant to tumor growth. Therefore, it must be either aiding or inhibiting tumor growth—or both. I will present arguments on both sides of this question.

The argument that the lymphoid infiltrate is inhibitory to the tumor depends, as far as I can see, on two sets of essential facts. The first fact is that lymphoid cells can, under the proper circumstances, be cytotoxic to their specific targets in vitro and can also specifically inhibit the growth of tumor transplants. Furthermore, in some virus systems immunity definitely inhibits oncogenesis (*8*). These facts need no elaboration.

The second set of facts is the series of observations that a lymphoid infiltrate is usually associated with a better prognosis. This association is apparent in cases of human breast cancer in which a heavy infiltrate is present (*30*). Furthermore, many of the premalignant skin lesions associated with a lymphocytic infiltrate never become overtly malignant. The apparent association of a lymphocytic infiltrate with a good prognosis would certainly seem, at first glance, to be a strong argument in favor of a defensive antitumor role for the infiltrate. Furthermore, it is generally believed that the defense of the body against antigenically distinctive invaders is the lymphocyte's raison d'etre.

The argument in favor of a tumor-stimulatory role for the lymphocytic infiltrate, although in my opinion strong, is less familiar and requires more elaboration. It depends on three points: 1) Under proper circumstances, either specifically immune or normal lymphoid cells, mixed with tumor cells, *can* stimulate growth rather than kill, and the proper circumstances probably often exist in the early in situ neoplasm (*7*). 2) Lymphoid cell depletion can sometimes inhibit, rather than enhance, tumor occurrence and/or growth (*6, 31*). 3) The good prognosis associated with lymphocytic infiltration is compatible with a tumor-stimulatory, rather than a defensive, role for the lymphocytes, a point I shall explain shortly.

The fact that under certain conditions the immune reaction can stimulate target cells appears to be well established, but the mechanisms remain obscure (*32-43*). Much of the work suggestive of direct immunostimulation of tumor cells is not absolutely conclusive, since the observations could sometimes be interpreted by alternative explanations. For example, I was able to show that immune spleen cells, when mixed at low ratios with target tumor cells, specifically stimulated tumor cell growth in Winn-type assays in immunodepressed mice (*32*). Although direct immunostimulation was the simplest explanation, it could be argued that spleen cells, when present in small numbers, expressed a disproportionately effective suppressor cell activity and so interfered with a small residual host immunity. In in vitro settings it is also possible to argue, though I think with some difficulty, that suppressor cells might be disproportionately activated when lymphoid cell:tumor cell ratios are low (*41*). That actual stimulation, rigorously distinguishable from any kind of suppressor cell activity or blocking effect, does indeed exist is best shown in the work of Shearer (*40*). This investigator has shown, in completely in vitro allogeneic systems, that antibody in low titer can specifically stimulate target cells, whereas in higher concentration the same serum is specifically cytotoxic. This observation makes it probable that the Winn-type data and the in vitro studies with lymphoid cells were also demonstrating actual tumor cell stimulation rather than, or in addition to, suppressor cell or blocking activity. The possibility is also raised that many or all of the effects described as "enhancement" by antiserum and studied in great detail by Kaliss and his associates in allogeneic systems were due to direct stimulation of the target tumor cells (*44*). It seems unlikely that all instances of immunostimulation are mediated by antibody, but I think this remains a possibility. It is also evident that many competing mechanisms, including suppressor cells, are probably at work confounding simplistic interpretations.

In work apparently demonstrating immunostimulation of tumor by immune cells or antiserum, several observations seem to have been common to many of the varied experimental designs. Seemingly most important was the effect of the relative concentrations of immune cells or antibody and the target cells. Characteristically, a biphasic result was seen in which low relative concentrations of immune cells and/or effectors produced stimulation, whereas higher concentrations were inhibitory or cytotoxic. In many experiments a strong nonspecific stimulation of the target cells was also seen, over and above the specific component of the response (*32, 45*). However, variations were great from tumor to tumor, the reasons for which remain unknown. Some data suggested that blood leukocytes harvested early after the implantation of an immunogenic tumor may have been specifically stimulatory, the same relative number becoming indifferent or inhibitory when harvested during a later period of tumor growth (*35*); perhaps this phenomenon is explicable in terms of the probably greater proportion of specifically immune cells harvested at the later periods.

Although purely quantitative variables seem to be of great importance in determining whether the effect of an immune reaction is stimulatory or inhibitory to target tumor cells, it is almost certain that important qualitative determinants also exist. The cellular composition of the leukocytic infiltrate is known to vary morphologically depending on the particular tumor. Gorer (*46*), who divided the types of infiltrate into three basic categories, believed that these types were associated with functional differences. Furthermore, the mast cell and eosinophil content of the infiltrate can vary widely. It is reasonable to suppose that the functional attributes of various forms of leukocytes, not recognizable morphologically, may vary greatly from infiltrate to infiltrate, and analysis of these variations is only just beginning (*47*). Although these qualitative variations in the infiltrate are undoubtedly very important, so little is understood about them that they cannot be considered in the development of the argument of this editorial.

A number of observations suggest a possible role of immunostimulation of tumor growth in actual oncogenesis as well as in a variety of transplantation phenomena. I have pointed out that clear evidence exists that depression of the immune mechanism by experimental means can often potentiate viral and, more debatably, chemical oncogenesis, but in view of the biphasic character of the lymphoid cell effect (discussed above), is this observation due to a decrease in tumor inhibition or to an increased level of stimulation? Furthermore, precisely the opposite is also true. For example, in the mammary tumor system of mice (a "weak" immunogenic system), immunocrippling inhibits rather than potentiates tumor formation (*6*). There is also some evidence, which needs further substantiation, that transplants of weakly immunogenic tumors may be inhibited in immunodepressed hosts (*9*). Others have observed that allogeneic and xenogeneic tumor transplants tend to grow more slowly, to be less invasive, and to metastasize less frequently in nude mice than in the syngeneic strain of origin (*48*). This is so despite the fact that athymic nude mice have no detectable resistance to the growth of allografts of normal tissues. Since the immunogenicities of chemically induced tumors are inversely correlated with the latencies of their formation and since the growth rates of these same primary tumors are also inversely correlated with their latencies, it can be deduced that the growth rates of primary tumors are directly correlated with their immunogenicities, although direct evidence of this latter correlation did not reach statistical significance (*15*). Thus the more immunogenic the primary tumor, the faster it tends to grow! A further argument in favor of a role for stimulation in tumor biology derives from the observation that tumors are constantly throwing variants of lesser immunogenicity (*49*). How is it then that most, but not all, tumors are immunogenic and remain so to varying degrees through numerous transplant generations (*49*)? Is immunogenicity sometimes maintained by immunoselection? All of these observations can be interpreted to mean that some degree of immunologic responsiveness may sometimes favor tumor growth, but in each instance alternative explanations of the observed phenomena are possible.

From what has been determined concerning the conditions under which immunostimulation of tumor growth is likely to occur, what can be said about conditions in the de novo tumor that has just reached a sufficient size to effectively signal its presence to the immune mechanism? As has already been discussed, this size is probably on the order of many thousands of cells, even in virus systems in which immunization may have occurred prior to the particular neoplastic transformation being considered. Initially the immune response must necessarily be small, even in such immunized hosts. Put another way, lymphoid cells will be attracted to the tumor at some finite rate—initially the number accumulated must be small. Thus early in the process, and for a certain indeterminate period of time, the number of lymphoid cells relative to the number of tumor cells must be low; these are the conditions in which stimulation rather than inhibition is likely. Furthermore, on examining the early neoplasm that has already achieved a heavy lymphoid infiltrate, I am struck by the fact that the infiltrate often tends to be concentrated outside the tumor, e.g., in the dermis in an incipient epithelial tumor of the skin. Few, if any, lymphoid cells may be discernible within the tumor per se or even in close contact. Thus the concentration of effector substances, antibody, or other lymphoid cell products is probably low within the tumor proper and, therefore, probably stimulatory rather than inhibitory. It is perhaps reasonable to suggest that the immobilization of lymphoid cells in the dermis may occur because of the release of diffusible antigen from the neoplasm (*50, 51*); the lymphoid accumulation might be analogous to the precipitin line in an Ouchterlony plate.

If one can judge by the analogy with the tuberculin reaction or the reaction to a skin allograft, most of the lymphoid cells attracted to the area of the tumor do not specifically recognize tumor antigens (*52*). In tests done with normal (i.e., not specifically immune) lymphoid cells, only target cell stimulation was usually seen, at least until the ratio of lymphoid cells to tumor cells became exceedingly large (*32, 45*). Thus even if the number of lymphoid cells around the tumor eventually becomes high, the infiltrate attracted to the tumor may be largely nonspecific and, therefore, may tend to remain stimulatory.

If the lymphoid infiltrate usually stimulates the tumor, why the good prognosis often associated with such an infiltrate? At least three, not mutually exclusive, explanations can be offered.

First, the lymphoid infiltrate may be a marker for a tumor of lesser malignancy rather than a cause of that behavior. There are many reasons to believe that the capacity to be stimulated to hyperplasia by a lymphoid infiltrate is a property that a tumor shares with its normal tissue of origin (*7*). Apart from the controversial literature concerning the possible stimulation of fetal growth by immunity (*7*), many examples exist in pathology of situations in which an immune reaction and/or a lymphoid infiltrate of one kind or another is associated with hyperplasia. In rheumatoid arthritis, the characteristic lesion often exhibits a tumorlike hyperplastic quality (*53*); the arterial smooth muscle proliferation in chronic allograft rejection has been often noted (*54*); and the association of lymphoid infiltration with epithelial hyperplasia in an insect bite is astounding (*55*). Numerous other such associations have been documented, including a role for lymphoid cells in compensatory hyperplasia in the liver and kidney (*56*). In fact the idea that the lymphocyte may be a growth promoter has a long and honorable history dating back to Carrel (*57*); it is not a new or novel idea. If the tumor shares with normal tissues the capacity to be stimulated by lymphocytes, it is reasonable to think of the early tumor that attracts lymphoid cells as "lymphodependent." Presumably this is a property that might, with time, be lost during the course of tumor progression, just as an estrogen-dependent mammary tumor may gradually lose its dependency on estrogen. Dependency of any kind is a characteristic property of the early tumor, although in some instances it may be retained indefinitely (*58*). In this context, it should be noted that in human lung carcinoma, the leukocytic infiltrate is more marked in the better differentiated tumors (*59*). Lymphoid dependence is thus a marker of a tumor still young in its biologic evolution—a tumor that will generally carry a better prognosis than will one that is biologically more advanced, i.e., less differentiated.

The second reason why lymphodependency is compatible with a good prognosis requires a further consideration of the neoplastic process as seen in skin. The preneoplastic skin lesions associated with a marked lymphocytic infiltrate, such as senile or actinic keratosis, probably represent clones of epithelial cells in which an antigenic change has occurred, and repeated episodes of compensatory hyperplasia have produced a lesion large enough to signal the immune reaction. If my thesis is correct, this reaction then serves to amplify and maintain the hyperplasia, not eliminate it, thus increasing the chance that the further changes necessary to full-blown neoplasia may eventually occur. Although a dermal lymphoid infiltrate may often be necessary to maintain a hyperplastic lesion in the epithelium, possibly the same infiltrate may simultaneously prevent the antigenic epithelial cells from metastasizing. Perhaps any epithelial cells that penetrate the dermis might find the concentration of effector substances and/or antibody too high and therefore be inhibited. There might thus be selection for neoplastic cells of less susceptibility to lymphoid effectors and with fewer antigens. Such cells might be the only type that could safely traverse the lymphoid area. In order to then leave the area of lymphoid concentration, such cells, now low in antigenicity, would also have to acquire the capacity to proliferate in the absence of the lymphoid cells, which they could not themselves attract. Thus a metastasis from a primary skin neoplasm would usually be expected, because of selection, to exhibit less immunogenicity than the primary in situ lesion and to be less lymphodependent. This expectation conforms to the observation that the lymphoid cell infiltrate is most marked in the lesions that are still largely in situ or still in the early stages of dermal invasion (*22*).

Of course, if my thesis has merit, there is a third and obvious reason why lymphodependency may be associated with a good prognosis. Lymphoid cells tend to be unusually sensitive to radiation and chemotherapeutic chemicals (*60*); perhaps these therapies are effective largely because of, not in spite of, their effects on the lymphoid infiltrate!

If the early lymphocytic infiltrate is really important in early tumor development, one important and seemingly contrary observation must be rationalized; the athymic nude mouse appears to be essentially not more susceptible or resistant to the occurrence of either carcinogen-induced or nonlymphoid spontaneous tumors than are heterozygous controls (*3, 61, 62*). This fact is a strong argument against either a stimulatory or an inhibitory role for the lymphocytic infiltrate. On the other hand, it seems, a priori, most unlikely that the heavy lymphocytic infiltrate seen in early carcinogen-induced tumors in normal rodents could be completely inconsequential.

The explanation of this seeming paradox could perhaps be contained in the previously discussed biphasic nature of the effect of immune lymphoid cells on their target. Let us consider a nearly total lack of lymphoid infiltrate as the baseline (i.e., the condition in the nude or newborn mouse).

Under these conditions tumor growth will be poor (*48, 63*); however, a small increase in the number of lymphocytes would produce accelerated tumor growth. This acceleration has actually been observed by Giovarelli et al. (*64*) and may explain the potentiation of chemical oncogenesis sometimes seen in partially immunocrippled or young animals (*65*). With increasing numbers of lymphocytes, i.e., the normal situation, the stimulation should give way to increasing inhibition and the incidence curve should again decline to the baseline and, perhaps, beyond (*64*). Therefore, the difference in oncogenic susceptibility might be difficult to detect between a nude mouse with little lymphocytic infiltrate and a normally reactive animal in which stimulation and inhibition of a primary untransplanted lesion might be nearly at the balance point. Thus the net effect of the infiltrate on the incidence of cancer might be small. However, the explanation is undoubtedly more complicated, since the nude mouse has recently been shown to possess a radiosensitive mechanism of tumor resistance (*66*).

Speculations along the lines I have been pursuing may explain a curious phenomenon often observed in animal experimentation, so-called "concomitant immunity" (*67*). The phenomenon consists of the observation that a primary tumor inoculum may persist and grow in an animal in which subsequent inoculations of the same tumor are inhibited. In fact, the growth of the primary inoculum may even be "enhanced" by the subsequent challenge (*68*). The survival of the primary inoculum is not a result of selection of resistant tumor cells, since it can be observed in successive transplant generations of the same tumor with no indication that the tumor has changed, as would be the case had selection occurred. The phenomenon can be seen in normal tissue allografts and in certain infections, especially those giving rise to granulomas. In both syphilis and tuberculosis, the organism is commonly found alive as a result of the primary infection at a time when there is immunity to superinfection (*69*). The same phenomenon occurs in some parasitic diseases (*70*). The trite explanation that a target tissue is more vulnerable to any resistance mechanism prior to establishment of a blood supply (the added insult to injury theory) might be applicable to tumor or tissue grafts, but hardly seems tenable for a parasite, nor would it account for the previously discussed, sometimes enhanced growth of a primary tumor graft.

Why is the larger primary colony of target cells relatively unaffected by a level of immunity capable, simultaneously in the same animal, of suppressing a smaller challenge inoculum? The cells of both colonies would supposedly be exposed to the same concentration of immune effectors. The answer may lie in the geometry of the situation. I propose, as previously discussed, that the target cells are releasing antigen into their immediate surroundings and that a concentration of antigen exists that immobilizes immune effector cells—lymphocytes and macrophages. A large colony will release more antigen than will a small one. Thus at some distance from the border of the large colony, leukocytes will be immobilized and/or antibody precipitated. Immune effector substances, lymphokines and/or antibody, will therefore be localized at some distance and be at low concentration in the large primary colony per se; they may, in the lesion proper, even be in the stimulatory rather than the inhibitory range of concentration. The small, secondary, challenge colony releases less antigen and is overwhelmed by the same concentration of immune effectors that is stimulatory to the primary colony; the leukocytes and/or antibody, rather than being immobilized at a distance, invade the colony to form an inhibitory concentration. In the larger colony, the volume of antigen-producing cells is larger relative to the surface area of the colony, where the interaction with lymphoid cells occurs, because of the surface-to-volume relationship of a sphere ($S = 4\pi R^2$, $V = 4/3\pi R^3$).

Why did the immune response evolve in a way that stimulates as well as inhibits? It seems to be of no obvious benefit to the host to stimulate the primary neoplasm. However, if stimulation is a necessary price to pay for the inhibition of tumor spread and metastasis, then the situation is understandable. If the immune response evolved from a more primitive growth-regulatory mechanism, the linkage of stimulation to inhibition might be basic and unbreakable. Alternatively, in the strategy of defense against bacteria and parasites, there might actually be an advantage in stimulating the first invaders. For common environmental pathogens, the odds are great that the first chance encounter would involve fewer invaders than some subsequent meeting. It would be to the host's advantage to become maximally immunized as a result of the first exposure. This might require that the first invasion be amplified to some extent to provide optimal and perhaps prolonged antigenic stimulation and to prevent "low-zone" tolerance. This strategy entails the risk that the first encounter might sometimes prove overwhelming as, in fact, it sometimes is. It is interesting in this context that tumors induced in athymic nude mice by the Moloney sarcoma virus do not regress as they do in normal hosts; however, the tumors grow initially more slowly in nude mice; this suggests that in normal animals the Moloney sarcoma virus tumors are perhaps stimulated by the early immune reaction (which is missing in nude mice) but inhibited by the later immune response (*71*).

In summary, I believe that the available evidence is compatible with the hypothesis that many tumors in humans and other animals pass through a stage, most notable early in their progression, when they are, to varying extents, lymphodependent. Furthermore, there are important quantitative effects such that the same lymphoid cell population that is stimulatory to tumor cells at low concentrations may be inhibitory at higher concentrations, a phenomenon that might sometimes limit the spread of a lymphodependent tumor. The commonly biphasic nature of the effect of an immune reaction on target tumor cells may contribute to the difficulty in demonstrating an effect of immunodepression on tumor induction in some tumor systems. The biphasic response also makes a rational attempt at immunotherapy difficult; in some cases the best result might be achieved by a reduction rather than an increase in immune reactivity. I would intuit, however, that the already successful tumor has, by selection, usually achieved an optimal (for the tumor) level of immune interaction with the host (*49*) so that an alteration toward either increased or decreased immunity might prove beneficial to the patient. At least it seems reasonable to hope that, under these circumstances, there may be little possibility that the patient will be harmed by attempts to alter the immune response and there may, on the other hand, be some chance of a beneficial result. The hypothesis that many human tumors are to some extent lymphodependent offers some hope of developing truly effective immunologic approaches to prevention and therapy—at least the prospect is much better than might be were most human tumors analogous to spontaneous rodent tumors and perhaps truly immunologically inert.

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GUEST EDITORIAL

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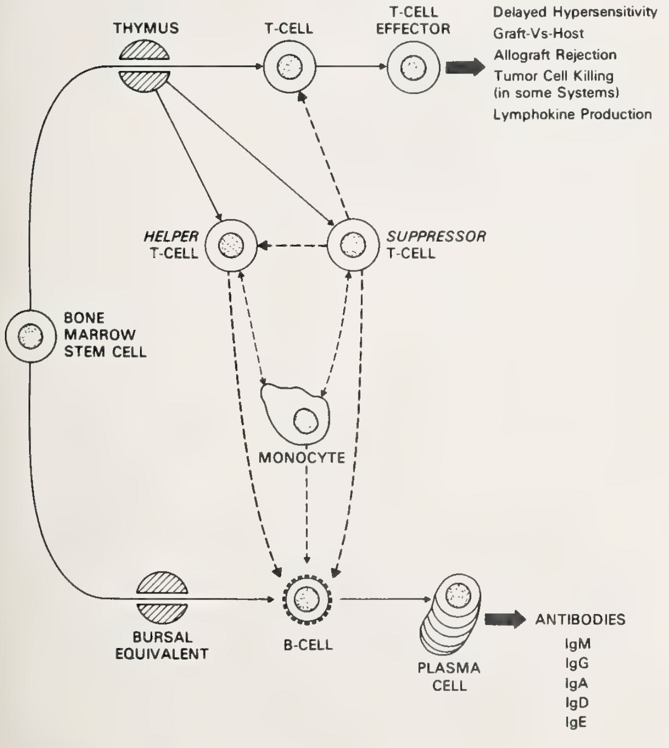
Suppressor Cells in Neoplastic Disease[3]

Immune responses require a complex interplay of negative as well as positive regulatory factors. A schematic representation of human lymphocyte maturation is presented in text-figure 1. Bone marrow lymphocyte precursors may develop according to two pathways of final differentiation. Cells which undergo a thymus-dependent process of maturation are called T-cells. T-cells mediate the classic reactions of cellular immunity, including delayed cutaneous hypersensitivity, graft-versus-host reactions, direct tumor cell killing in some systems, and lymphokine production. Precursor cells that undergo a bursal equivalent pathway of differentiation are referred to as B-cells. B-cells are the clonal progenitors of plasma cells, which are committed to the secretion of immunoglobulins. B-cells express surface membrane immunoglobulin with the same antigen-binding specificity as the immunoglobulin finally secreted by their clonally derived plasma cells.

Immune responses resemble other complex biologic processes such as hemostasis, complement activation, and hormone regulation because they are governed by a series of positive as well as negative regulatory factors (*1–3*). In many systems, the final transition of B-cells into immunoglobulin-secreting plasma cells requires the cooperation of helper T-cells and macrophages (*4, 5*). Similarly, under certain conditions, the ultimate transition of B-cells into immunoglobulin-secreting plasma cells may be inhibited by suppressor T-cells and macrophages.



TEXT-FIGURE 1.—Schematic representation of cell regulatory interactions in the differentiation of human lymphocytes. *Interrupted arrows* indicate some, but not all, targets of regulatory control.

Suppressor cells participate in a number of critical immunologic phenomena and thus have an important role in the phenomena of immunologic tolerance and antigenic competition. Such cells also play a crucial part in the control of contact or delayed hypersensitivity reactions, in the genetic regulation of immune responses, in the perpetuation of allotype suppression, and in the limitation of antibody responses to a wide variety of antigens. Suppressor cell regulation of immune responses involves an intricate network of different systems rather than one simple system. Essentially every major cell type that promotes immune responses may also inhibit them. Thus certain classes of B-cells (*6–8*), monocytes (*9–11*), and especially T-cells (*2, 3, 12*) may play a suppressor role for different phases of immunity.

Some suppressor regulatory functions are antigen- or carrier-specific, whereas others are nonspecific (*2, 3*). Suppressor T-cells may regulate the production of all immunoglobulin classes (*3, 13, 14*), a single immunoglobulin class (*15–17*), a single immunoglobulin allotype (*18, 19*), or even a single immunoglobulin idiotype (*20, 21*). Certain classes of suppressor cells may influence cellular immune functions alone without an appreciable effect on humoral immunity; furthermore, suppressor cells may work on the afferent or efferent limb of the immune response (*22–24*). The purposes of this review are 1) to characterize the role of suppressor cells in the immunologic enhancement of tumor growth

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ABBREVIATION USED: ATS=anti-mouse thymocyte serum.

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

in certain systems, 2) to describe the alterations of suppressor cell function that may cause or perpetuate the immunodeficiency of certain tumor-bearing hosts, and 3) to discuss the implications of suppressor cell function for further clinical investigation and future therapy of human neoplasia.

SUPPRESSOR CELLS IN THE POTENTIATION OF TUMOR GROWTH

In virtually all cases that have been carefully studied, neoplastic transformation is accompanied by the appearance of tumor-specific neoantigens on malignant cells. Host defense cells frequently recognize these as "foreign" surface antigens and, under certain circumstances, can inhibit tumor growth. However, in many cases the tumor progresses in spite of its "foreign" antigens and an apparent host immune response. For years, the ineffectual immune response by tumor-bearing hosts was ascribed to the presence of circulating, blocking factors thought to impede the destruction of tumor cells by host defense cells (*25*). Originally, some workers classified such blocking factors as serum antibodies (*25*). However, antibody fragments (*26, 27*), soluble antigens shed by tumor cells, or antigen–antibody complexes (*28, 29*) might all have a function in enhancing tumor growth.

More recently, it has been proposed that host suppressor cells and their factors mav undermine an effective antitumor immune response and lead to immunologic enhancement of tumor growth. In some studies, suppressor cells inhibited so-called natural killer cells, which have been postulated to play a role in the elimination of early leukemias, lymphomas, and other tumors (*30*). Other studies have shown a paradoxical reduction in the host's resistance to tumor by treatments that may superficially resemble immunization. One carefully studied tumor model involves the use of 3-methylcholanthrene-induced sarcomas transplanted in A/J mice (*31*). One of the mechanisms favoring tumor growth is the development of specific suppressor T-cells (*31–35*). In this system, tumor cells implanted into normal mice killed the host within 40 days, whereas tumor cells transplanted to mice previously rendered immune to this tumor were rejected in approximately 2 weeks. (Mice highly immune to the syngeneic tumors were prepared by complete surgical excision of tumor 1 week after sc injection of 1×10^6 tumor cells followed by several additional immunizations with tumor cells.) When thymus or spleen cells from tumor-bearing hosts were infused iv into *immune* mice at the same time that the mice were challenged with live tumor cells, tumor growth was enhanced. Thus these thymus and spleen cells from hosts with growing tumors contained suppressor cells. Furthermore, such suppressor cells could counteract host defense responses when injected into immune mice during a time of ongoing tumor rejection. These suppressor cells remained in force for as long as tumor growth was active. However, suppressor cell activity disappeared promptly after extirpation of the tumor.

The immunosuppressive activity of lymphoid cells from tumor-bearing hosts was totally abolished by in vitro treatment with antitheta serum or rabbit ATS. These studies were extended by testing the effect of in vivo ATS administration on the growth of primary transplantable syngeneic tumors. Repeated administration of ATS after tumor cell inoculation resulted in a marked inhibition of primary tumor growth. This inhibition of tumor growth probably occurred because immunosuppressing T-cells (which were interfering with the capacity of host effector cells to check tumor growth) were eliminated. It is important to emphasize that the suppressor T-cells involved in the enhancement of tumor growth in this system were specific in their activity and did not affect the growth of unrelated tumors.

In further studies, a soluble factor with identical tumor-enhancing activity was extracted from the lymphoid cells of tumor-bearing hosts. This soluble factor was obtained by snap freezing of thymus cells followed by centrifugation at 100,000×*g*. The activity was sensitive to pronase but not to RNase. It had a molecular weight of less than 70,000 daltons. It did not react with anti-Fab reagents, but was specifically absorbed by tumor cells. Of great interest was that the suppressor activity was removed by antisera to appropriate *H-2* determinants.[4]

This example of a tumor-specific suppressor T-cell factor and other specific soluble T-cell factors capable of inhibiting humoral immune responses or contact sensitivity have intriguingly similar biologic and immunochemical properties (*12, 36–38*). Inasmuch as suppressor T-cells and certain specific soluble suppressor factors derived from such cells contain antigens encoded in the *I-J* subregion of the *H-2* complex in mice, antibodies directed against such *I-J* determinants would be expected to nullify suppressor activity in vivo. Recent studies have, in fact, shown that alloantisera directed against appropriate *I-J* antigens cause significant enhancement of IgM and IgG immune responses to sheep erythrocytes (*39*). On the basis of these observations, Greene et al. (*40*) tested the hypothesis that in vivo administration of antisera directed against *I-J*-coded determinants would eliminate suppressor T-cells from tumor-bearing mice and cause an inhibition of syngeneic tumor growth. They found a highly significant retardation of tumor growth in mice after daily iv treatment with *microliter quantities* of anti-*I-J* serum. Spleens from tumor-bearing mice treated with anti-*I-J* antisera no longer contained specific suppressor cells. These experiments provide elegant proof that tumor-specific suppressor T-cells take part in actually protecting certain neoplasms against host immune defenses. In addition, these experiments provide a model for the specific serologic removal of suppressor T-cell activity. As a consequence of this selective suppressor T-cell removal, the host is able to mount a more effective immune response against lethal tumors.

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[4] *H-2* determinants represent gene products of the major histocompatibility complex in the mouse.

Other workers have provided additional data to support the concept that T-lymphocytes from tumor-bearing mice may suppress host resistance to syngeneic tumors. For example, spleen cells from mice with Lewis lung carcinoma enhanced tumor grafts when injected into mice either before tumor challenge or as a mixture with the tumor inoculum (*41, 42*). Removal of T-lymphocytes by treatment of spleen cells with antitheta antisera reduced this enhancement of tumor growth. Also, adult thymectomy before tumor inoculation has led to a reduction in the number of metastases seen in Lewis lung carcinoma. Further evidence in mice would suggest that in certain instances, carcinoma growth can be inhibited by administration of hydrocortisone 3-5 days after inoculation of tumor cells (*43*). Hydrocortisone may eliminate precursors of suppressor cells, and the overall deficiency of mature suppressor cells that secondarily occurs allows the host to mount an increased cytotoxic response against the tumor.

In mice, the generation of cell-mediated toxicity against syngeneic tumor cells in vitro can be suppressed by a subpopulation of nylon-adherent T-cells (*44*). The overall concept that nylon-adherent T-cells may suppress tumor-specific cytotoxic responses in vitro has been extended to humans. The peripheral blood of patients with osteogenic sarcoma simultaneously contains tumor-specific cytotoxic cells and suppressor cells (*45*). Unfractionated circulating lymphocytes from 12 of 28 patients with osteogenic sarcoma were cytotoxic to osteogenic sarcoma cells in vitro. When lymphocytes from those patients whose unseparated cells were not cytotoxic underwent fractionation on bovine serum albumin gradients, a tumor-specific cytotoxic cell subset was unmasked in 11 of 13 patients. Lymphocytes capable of suppressing the tumoricidal activity of autologous cytotoxic lymphocytes were found in 4 of 10 patients tested. The cells responsible for this suppressor effect formed spontaneous sheep cell rosettes and adhered to nylon. The suppressor cell population found in patients with osteogenic sarcoma was generally not observed in the normal population or in parents of the patients. Of the 4 patients who had suppressor cells, 3 had pulmonary metastases. The fourth patient developed pulmonary metastasis shortly after such suppressor cells were detected. Only 1 of 6 patients without suppressor T-cells was reported to have metastatic disease. These observations may be early clues that suppressor cells can affect the clinical presentation of patients and the final outcome of their malignant neoplasms.

Recent reports have described the presence of non-T suppressor cells in tumor-bearing hosts (*6, 10, 14, 46*). Such suppressor cells appear to inhibit a series of immune responses in a relatively nonspecific fashion. Gorczynski (*6*) and Kirchner et al. (*10*) have demonstrated suppressor cells in spleens of mice bearing progressive tumors induced by murine sarcoma virus. These suppressor cells inhibited lymphocyte stimulation by mitomycin C-treated syngeneic lymphoma cells and also impaired the migration-inhibition response of immune cells to murine sarcoma viruses. The non-T suppressor cells also inhibited lectin-induced DNA synthesis by syngeneic normal spleen cells. Thus they may inhibit T-cell proliferative responses nonspecifically. Both groups of investigators showed that the cell doing the suppressing is not a T-cell. However, whereas Gorczynski (*6*) concluded that the suppressor cell was a B-cell, Kirchner et al. (*10*) believed that it was a macrophage. Other investigators studying progressively growing 3-methylcholanthrene-induced tumors also demonstrated non-T suppressor cells in tumor-bearing mice (*47*). In these studies, cells from tumor-bearing mice were capable of nonspecifically suppressing the capacity of lymphocytes from normal mice to be immunized in vitro against transplantation alloantigens. These cells from tumor-bearing mice were removed by a passage through nylon but not by anti-theta treatment. Thus suppressor cells of non-T-cell origin are commonly seen in tumor-bearing animals and may nonspecifically suppress a variety of immunologic functions. Such non-T suppressor cells have been described in humans and may have a function in the progressive growth of some tumors, or at least in the overall immunodeficiency state associated with certain cancers. This possibility will be discussed further.

SUPPRESSOR CELLS IN THE IMMUNODEFICIENCY ASSOCIATED WITH NEOPLASTIC STATES

Certain neoplasms, particularly multiple myeloma (*48-50*) and the syndrome of thymoma plus hypogammaglobulinemia (*51*), may be associated with abnormalities of humoral immunity that may lead to lethal infections. It is known that suppressor cells may have a role in the pathogenesis or perpetuation of common variable hypogammaglobulinemia (*13, 52*). Similarly, suppressor cells have been implicated in the polyclonal immunodeficiency associated with multiple myeloma (*14, 46*) and in the anergy associated with Hodgkin's disease (*53, 54*), as well as in widespread fungal infections (*55*).

A significant percentage of patients with thymoma (especially of the spindle-cell variety) have hypogammaglobulinemia and recurrent infection. Circulating suppressor cells may be observed in a number of patients who have hypogammaglobulinemia (*56*). Suppressor cells found in this thymoma syndrome appear to be T-cells. However, suppressor T-cells may not be the primary factor leading to humoral immunodeficiency in all cases.

Patients with multiple myeloma have significantly reduced in vivo polyclonal immunoglobulin synthesis rates and, consequently, they have low serum levels of polyclonal immunoglobulins (*57*). They may also have reduced percentages of circulating lymphocytes with normal surface-membrane immunoglobulin determinants (*14*). Some patients have an increased incidence of infection with highly pathogenic bacteria even before the underlying myeloma is diagnosed.

A number of theories have been constructed to explain the impaired B-lymphocyte function in myeloma. According to one theory, myeloma tumor cells release infective cell units of RNA that may alter the expression of normal immunoglobulin receptors on the surfaces of host B-lymphocytes, thereby impairing the recognition of foreign antigens and the appropriate antibody responses (*58*). According to another theory, myeloma cells release specific mitotic inhibitors (so-called chalones), which block the critical expansion of normal B-lymphocyte clones in response to antigenic challenge (*59*).

In the past, most workers have focused on myeloma cells or their direct products as the cause of immune impairment in myeloma. We analyzed the role of suppressor cells in the pathogenesis of this immunodeficiency by applying an in vitro immunoglobulin biosynthesis assay to the study of peripheral blood lymphocytes from patients with multiple myeloma (*14, 60*). We found that the lymphocytes of myeloma patients showed profound depression of polyclonal immunoglobulin production in vitro. In addition, peripheral blood mononuclear cells from over half the myeloma patients tested suppressed the polyclonal immunoglobulin production of normal lymphocytes cocultured with them. These suppressor cells were extremely radioresistant, which indicated that monocyte- or macrophage-like cells might be responsible for the suppressor phenomenon observed. Removal of phagocytic mononuclear cells nullified the suppressor activity and, in one case, augmented the capacity of the remaining peripheral blood mononuclear cell to synthesize immunoglobulin. Malignant plasma cells did not appear to be *directly* responsible for the suppressor effect observed. These observations suggest that at least one mechanism for the humoral immunodeficiency observed in myeloma patients is a block of polyclonal lymphocyte maturation by host regulatory phagocytic mononuclear cells (most likely macrophages). Other investigators have also observed that suppressor cells of non-T origin may play a role in the immunodeficiency state associated with multiple myeloma and certain mouse models of this disease (*46, 61*).

Our work in human myeloma is compatible with recent work in BALB/c mice bearing ascites plasmacytomas capable of inducing hypogammaglobulinemia (*62*). A factor extractable from malignant ascites cell populations suppressed immunoglobulin production by pokeweed mitogen-stimulated human and mouse lymphocytes. In contrast, similar extracts prepared from ascites cells that had first been depleted of macrophages failed to show suppressor activity, whereas extracts derived from glass-adherent cells were fully suppressive. Suppressor activity was associated with a low-molecular-weight protein. The study of such soluble suppressor factors may yield information useful in the therapy of patients with multiple myeloma and humoral immunodeficiency.

Patients with a variety of solid tumors may have impaired cellular immunity as assessed by delayed hypersensitivity skin reactions to such agents as streptokinase, streptodornase, and *Candida*. They may also show impaired contact sensitivity to 2,4-dinitro-1-chlorobenzene. These depressed reactions generally correlate with large total-body tumor burdens. In vitro lymphocyte transformation caused by nonspecific mitogens or specific antigens is also depressed in many such patients. It has recently become apparent that at least one mechanism for the depression of cell-mediated immunity in certain patients with advanced solid tumors may be nonspecific inhibition of T-cell function by circulating suppressor monocytes (*63*). In a smaller number of cases, suppressor T-cells may also play a role.

Hodgkin's disease is a particularly interesting lymphoma associated with an acquired in vivo and in vitro disorder of cell-mediated immunity. Some patients with Hodgkin's disease have an impairment of cellular immunity that appears far out of proportion to the detectable total-body tumor burden. It has been suggested that excessive suppressor cell activity may be one of the factors responsible for the immunodeficiency state associated with Hodgkin's disease (*53, 54*). In a careful study, irradiated leukocytes from 16 of 30 patients with Hodgkin's disease stimulated subnormal levels of tritiated thymidine incorporation in the presence of allogeneic lymphocytes from normal individuals (*53*). Since increasing the number of cells from patients with Hodgkin's disease led to still lower responses, the patients' cells probably suppressed the mixed-leukocyte reaction. This inhibitory activity of the patients' mononuclear leukocytes was corrected when cells adherent to glass wool were removed in advance. However, when phagocytic cells were removed (with the use of a method that provided minimal opportunity for adherence), the suppressor phenomenon remained, suggesting that the inhibitory cells were lymphocytes (or conceivably nonphagocytic monocytes). It is important to add that this suppressor effect may be aggravated by the presence of granulocytes at physiologic concentrations, because it is easy to overlook an ancillary role for granulocytes, platelets, and other hematopoietic elements in the regulation of immune function.

It has recently been suggested that the in vitro hyporesponsiveness found in certain patients with Hodgkin's disease may be due to the excessive production of prostaglandin E_2 by glass-adherent mononuclear suppressor cells (*54*). Furthermore, indomethacin, a prostaglandin synthetase inhibitor, was able to restore in vitro blastogenic responses with the use of lymphocytes from certain patients with Hodgkin's disease. These observations may eventually have important clinical relevance by providing a relatively safe pharmacologic basis for manipulating suppressor cell activity in selected disease states.

A small subset of patients with acute lymphoblastic leukemia have profoundly depressed serum immunoglobulin levels prior to therapy (*64*). Some patients who develop leukemias or lymphomas of T-cell origin probably show hypogammaglobulinemia because their tumors originate from a subclass of T-cells genetically committed to mediate suppressor function. In fact, we have recently analyzed the suppressor cell activity of neoplastic T-cells from an unusual child with acute lymphoblastic leukemia and hypogammaglobulinemia (*65*). The leukemic T-cells from this child acted as potent suppressor cells in an in vitro system. Systemic chemotherapy was followed by a transient restoration of the patient's serum immunoglobulin levels. One interesting feature of these leukemic cells is their apparent requirement for a cooperating subset of *normal* T-cells before a full suppressor effect can be expressed. This observation may indicate that a T-T interaction is necessary in the full generation of human suppressor effector cells in the normal immune network.

The recognition of a suppressor cell leukemia underscores the principle that neoplasms of T-cell origin may in some cases retain immunoregulatory properties. It is already known that leukemic cells from some, but not all, patients with the Sezary syndrome appear to retain helper-like activity (*66*). Various kinds of suppressor cell leukemias or lymphomas probably will be discovered in the near future. Patients with functioning neoplasms of suppressor cell origin might fall into an exceptionally poor prognostic category because the malignant cells could subvert host immune responses against both the neoplasm itself and pathogenic microorganisms.

IMPLICATIONS OF SUPPRESSOR CELL FUNCTION FOR FURTHER CLINICAL INVESTIGATION AND FUTURE THERAPY OF HUMAN NEOPLASIA

An understanding of suppressor cell function may provide new insight into the way some therapeutic modalities cause tumor regression. Chemotherapy, radiation, and surgery might, in certain cases, benefit tumor-bearing patients by an indirect effect on suppressor cells as well as the obvious direct effect on the tumor itself.

Most current chemotherapy research focuses on direct tumoricidal activity. The side effects that any given agent may exert on lymphoid elements are traditionally viewed as an unavoidable price for tumor cytotoxicity. However, data from a number of different laboratories suggest that, under the proper circumstances, a variety of conventional chemotherapeutic agents may actually augment host immunity. For example, if antigen is given 7 days after the completion of a 1-week course of 6-mercaptopurine, the humoral immune response is significantly potentiated compared to that of controls (*67*). This increase may be due to the selective inactivation or elimination of suppressor cells. Indeed, one of the most clinically versatile chemotherapeutic agents available—cyclophosphamide—has been proved to eliminate suppressor cell activity in several experimental systems, which could explain how this drug may actually increase some immune responses (*68–73*). Similarly, ionizing irradiation may potentiate certain in vivo immune responses by eliminating immunoregulatory suppressor cells (*70, 74–76*). Moreover, the removal of suppressor cells is thought to be the reason that irradiation of the host before tumor transplantation can retard the growth of lethal tumors in certain murine models (*77*). Finally, it is important to emphasize that suppressor cells in certain tumor systems may be relatively short-lived (*31*). In some cases, such suppressor cells either lose their functional activity or undergo cell death promptly after surgical removal of gross tumor. Thus even direct surgical intervention could conceivably have the hidden benefit of altering immunoregulatory balance in favor of the host without completely eradicating the neoplasm.

In the search for new agents and new overall strategies in the treatment of human cancer, modalities that eliminate suppressor cell activity might be worthy of high priority. It is extremely important to learn whether human suppressor T-cells express antigenic markers analogous to the *I-J*-coded products found in mice. If so, development of antibodies directed against the *I-J*-like determinants, capable of eliminating undesirable suppressor T-cells from tumor-bearing patients and thereby promoting tumor rejection (as has already been discussed in mice), might be feasible. In those patients with an apparently generalized immunologic hyporesponsiveness as a result of tumor growth, it may soon be possible to pharmacologically block the production of immunosuppressing factors. Highly promising, but as yet preliminary, observations with the use of indomethacin have already been discussed for patients with Hodgkin's disease (*54*). Furthermore, indomethacin has been used to partially restore in vivo cell-mediated immunity in a patient with acquired hypogammaglobulinemia (*78*).

Finally, it is urgent to understand how various commonly used immunotherapeutic agents such as *Corynebacterium parvum* or BCG may affect the cellular control of tumor surveillance and tumor cytotoxicity in cancer patients. To date, immunotherapy has made relatively little impact in the treatment of most human cancers (*79*). This failure may result in part from the capacity of conventional immunotherapeutic agents to activate suppressor cells as well as host cells engaged in checking neoplastic proliferation. For example, Kirchner et al. (*80*) have shown that injection of *C. parvum* into mice may cause a depression of specific antitumor cell immunity through the activation of macrophage-like suppressor cells. It is important to reemphasize that immunostimulation per se is not always desirable in treating cancer. Also, nonspecific immunotherapy that is effective in retarding *local* tumor growth (with the use of an agent such as BCG) will not automatically result in improved *systemic* immunity against the same tumor. In fact, actual enhancement of a second tumor graft has been observed after inhibition of the first tumor graft with BCG (*81*). The clear message is that nonselective immunostimulation may actually contribute to tumor growth under some circumstances or may aggravate an existing state of serious immunologic impairment in the defense against pathogenic microorganisms. A rigid reliance on such nonselective regimens could inhibit significant progress in immunotherapy as practical cancer treatment. However, we have reason to believe that new immunotherapeutic strategies, which incorporate recent insights into the suppressor cell system and the cellular control of immunity, can be designed. Such new strategies might make it possible to treat cancer patients by selectively activating host cells involved in the defense against neoplastic growth while neutralizing suppressor cells that impede effective immune responses.

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GUEST EDITORIAL—*by* **Herbert Z. Kupchik,** *Ph. D.,* **Norman Zamcheck,** *M.D.,* *and* **Calvin A. Saravis,** *Ph. D.*[1, 2]

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Immunochemical Studies of Carcinoembryonic Antigens: Methodologic Considerations and Some Clinical Implications [3]

IN 1965, Gold and Freedman (*1*, *2*) described carcinoembryonic antigen (CEA) of the human digestive tract as an antigen "specific" for adenocarcinomas of the human digestive tract and for digestive organs of human 2- to 6-month fetuses. Subsequently, the Montreal group (*3*, *4*) characterized CEA as a glycoprotein of 200,000 mol wt with β electrophoretic mobility. In 1969, they described a radioimmunoassay for measuring CEA in the sera of patients and reported positive levels (defined as $\geq$2.5 ng/ml of serum) in 35 of 36 patients with adenocarcinoma of the colon and insignificant levels in the sera of patients with nongastrointestinal malignancies or benign gastrointestinal diseases (*5*). These findings, plus the fall in circulating CEA in 5 patients after complete surgical removal of the tumor, offered the promise of a useful diagnostic procedure for patients with digestive system cancer. In 1970, using Thomson's procedure (Farr assay) (*5*) and antigen and antisera prepared in Montreal, we for the first time *a*) confirmed the presence of CEA in the serum of patients with carcinoma of the colon, *b*) showed a high incidence of positivity in patients with carcinoma of the pancreas, and *c*) demonstrated a significant number of elevated CEA values in patients with some nonmalignant diseases (*6*).

Several serum and plasma assays for CEA are now being evaluated. The assay findings (and thus the clinical interpretations) differ somewhat from those originally reported by the Montreal workers. Since these differences may depend in part on variations in the methods and reagents used, the purpose of this report is to summarize and discuss the various methods and reagents and to suggest how the complexity and variability of these factors may influence their clinical application. We shall discuss *a*) variations in CEA assay procedures, *b*) differences in antigen and antisera preparations, *c*) CEA-like substances, *d*) the presence of circulating anti-CEA antibodies, and *e*) the relationship of CEA to blood group antigens.

Assays for CEA

Comparisons.—The Farr assay, the Hansen zirconyl-gel (Z-Gel) assay (*7*), first used clinically by LoGerfo et al. (*8*), and the double antibody assay, initially described by Egan et al. (*9*) and modified by Laurence et al. (*10*), are the 3 most clinically studied tests. Similar in principle (table 1), all are inhibition assays in which the antigen in the test sample inhibits the binding of subsequently added, radiolabeled CEA to anti-CEA antibody. The modified double antibody test differs from the Farr and Z-Gel assays in several respects: *a*) It utilizes 0.2 ml serum or plasma, in contrast to the 5 ml serum and 1 ml plasma in the Farr and Z-Gel assays, respectively; *b*) it is a "direct" assay in that perchloric acid (PCA) extraction is not used as an initial step, as with the 2 other tests, thereby eliminating the requirement for subsequent dialysis and lyophilization; and *c*) a second heterologous antibody (anti-IgG antiserum) is used to separate out the CEA-anti-CEA complexes by precipitation of the IgG anti-CEA antibody. In the Z-Gel assay, zirconyl-phosphate gel probably precipitates the CEA-IgG anti-CEA complex at its isoelectric point; but, in the Farr assay, ammonium sulfate is used for the nonspecific precipitation of immunoglobulins.

Studying the modified Farr (*see* below) and Z-Gel assays, we found no significant qualitative differences (comparing positive and negative values using 2.5 ng CEA/ml serum or plasma as the cut-off) in 85% of

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Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

TABLE 1.—*Comparison of some clinical immunoassays for CEA*

Investigators	Extraction	Coprecipitation	Requires*	Normal level, ng/ml
Thomson et al. (*5*)	1.0M PCA	50% AmSO$_4$	5 ml serum, 5 days	<2.5
Hansen et al. (*7*)	1.0M PCA	Zirconyl-PO$_4$-gel	5 ml serum, 2 days	<2.5
LoGerfo et al.†(*8*)	0.6M PCA	Zirconyl-PO$_4$-gel	1 ml plasma, 2 days	<2.5
Go et al.†(*11*)	None	Zirconyl-PO$_4$-gel	0.1 ml plasma, 2 days	<2.0
Egan et al.(*9*)	None	Anti-IgG antibody	0.2 ml buffer, 1 day	0–2.5‡
Laurence et al. §(*10*)	None	Anti-IgG antibody	0.2 ml plasma, 2 days	<12.5∥
McPherson et al.(*12*)	None	Tube solid phase	1 ml serum or plasma, 5 days.	<2.5
Lange et al. (*13*)	0.55M PCA	Hemagglutination inhibition¶	2.5 ml serum, 1 day	<5.0

*Volumes shown are for single determinations (duplicates usually performed, double amount required).

†Modifications of Hansen's Z-Gel assay.

‡No clinical data reported. Normal levels reported by Coligan et al. (*14*).

§Modification of Egan's double antibody assay.

∥ Normal range for blacks is up to 30 ng/ml.

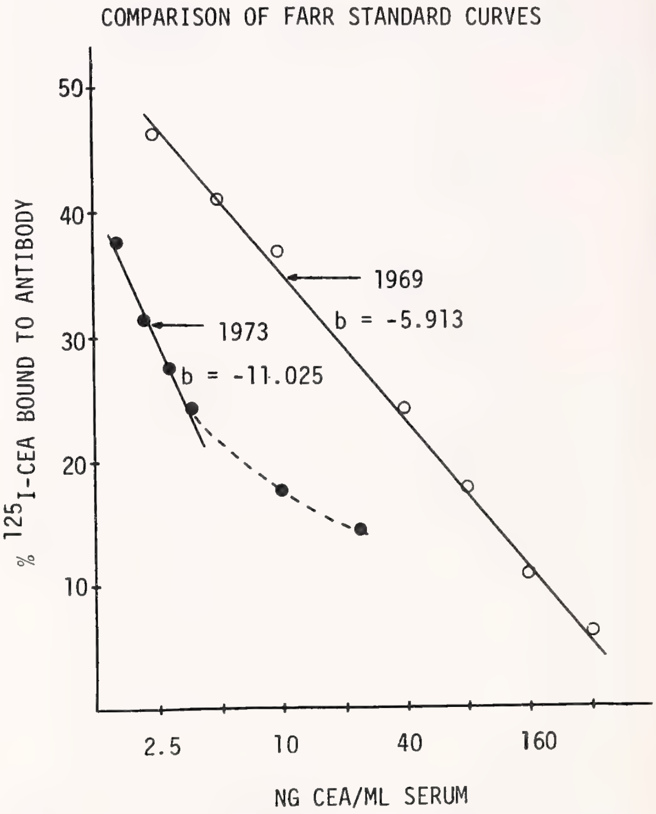
¶Nonisotope technique.

568 comparisons of 717 assays of samples obtained from 291 patients (*15, 16*). Laurence et al. (*10*) similarly compared the modified double antibody and Z-Gel assays and found 72% qualitative agreement among 272 tests. The reasonably good correlations between the different assays in several laboratories should encourage further tests and comparisons to elucidate the factors contributing to the observed disagreements.

Modification of the Farr assay.—Minor modifications in the original Farr assay used in this laboratory and in others (*17*) increased its sensitivity. The modifications included *a*) changes in the ratio of ^{125}I to CEA during radiolabeling, *b*) different goat anti-CEA antisera, and *c*) different incubation conditions.

This is reflected in the varied standard curves now obtained in this laboratory and those previously described by Thomson et al. (*5*) (text-fig. 1). The slope of the present curve is much steeper than that originally described ($b=-11.025$ vs. $b=-5.913$). As little as 1 ng CEA/ml serum is now measurable in the working range (the linear part) of present curves compared to 2.5 ng/ml in the previous curves. However, a consequence of the steep slope is the narrow working range (1–4 ng/ml) afforded when the curve is linearized as shown. Previously, values up to 320 ng/ml could be measured. To assess adequately changes in the high CEA levels of some patients with metastatic cancer undergoing therapy, a larger range is needed. This is achieved by dilution and reassay of samples containing >5–10 ng/ml. Alternatively, computer programs can be designed for mathematical expression of the nonlinear curves, which allow measurement of values up to 25 ng/ml without dilution. Egan et al. (*9*) described a computer program for rapid calculation of the results from the double antibody assay; the results from the Z-Gel assay were calculated by computer by Hansen.

Increased sensitivity of the Farr assay permitted detection of CEA levels <2.5 ng/ml and enhanced the ability to interpret clinically values around this level. An important consequence was the identification of low but significant amounts of CEA in patients with benign diseases, such as cirrhosis of the liver (*18*), ulcerative colitis (*19*), and nongastrointestinal tract cancer (*20*). Recently corroborating these findings, the Montreal General Hospital group in a collaborative study with 5 other centers (*17*) reported positive CEA levels in the sera of patients with carcinoma of the breast, endometrium, and bronchus as well as in ulcerative colitis, diverticulitis, and alcoholic cirrhosis;



TEXT-FIGURE 1.—Comparison of standard inhibition curves as described by Thomson et al. (*5*) and those presently obtained in this laboratory. The percent of radiolabeled CEA bound to anti-CEA antibody is plotted against increasing amounts of CEA added to 5 ml portions of normal human serum; *b* indicates the respective slopes calculated by linear regression analysis and shown as *solid lines* (the 1973 values at 10 and 25 ng/ml being omitted from the calculations).

they used the Farr assay and reagents prepared in Montreal.

Other investigators using the Z-Gel technique (*7*) demonstrated positive CEA levels in patients with cancer of the lung, breast, ovary, urinary bladder, and prostate and with neuroblastoma, multiple myeloma, and osteogenic sarcoma (*8*, *21*). Using the modified double antibody assay, Laurence et al. (*10*) also reported similar results.

Normal levels.—Normal levels of serum or plasma CEA may differ for each assay, depending on the method and the standard curves used. Using the Farr assay, this laboratory obtained >300 negative CEA assays (<2.5 ng/ml) on 60 normal individuals; using the Z-Gel method, we obtained >80 negative CEA assays on 25 normal individuals. All these normal individuals were <40 years old. When the Farr and Z-Gel tests were set up in this laboratory, normal sera and plasma obtained from the laboratory personnel were defined as "zero" controls (0 ng CEA/ml) by Gold and Hansen, respectively. Increasing concentrations of purified CEA were added to portions of normal serum or plasma to construct standard curves for the respective assays. Thus the CEA content of normal serum and plasma was blanked out. Subsequent normal sera and plasma have been compared against these initial standards to maintain a consistent "zero" baseline. Unless such consistent baselines are maintained, the values obtained by different tests will vary (and hence the clinical interpretation). Although the Farr method is still performed in this manner, many laboratories now use a Versene buffer (instead of normal plasma) to construct the standard curve for the Z-Gel assay. In this laboratory, the Versene curve is adjusted to conform to a zero normal human plasma baseline for consistency and comparison with the Farr assay. Test samples quantitated against the Versene buffer standard curve may be somewhat higher than those determined by the same standard curve adjusted to the zero normal plasma baseline. This difference is one reason that the use of a strict cut-off level of 2.5 ng/ml between "normal" and elevated is no longer universally acceptable. Laboratories using the Versene standard curve for the Z-Gel assay must adjust their cut-off to a comparable level (say, 4–5 ng/ml) or use ng/ml ranges when they report results obtained from patients. Of 1425 assays on healthy subjects of a wide range of ages done by the Hoffmann-La Roche Laboratories (*22*), 11.5% had CEA values >2.5 ng/ml of plasma while only 1.3% had CEA values >5 ng/ml when the Versene curve was used.

Coligan et al. (*14*) stated that normal serum had an apparent CEA content of about 2.5 ng/ml or fluctuated around 0 ng/ml, depending on whether the double antibody assay was referred to a buffered saline or normal serum standard curve. They reported no clinical observations. Using buffered saline standard curves in the clinical application of their modified double antibody assay, Laurence et al. (*10*) found in normal male and female Caucasians a plasma CEA content of 6–10 ng/ml. These high normal values result, at least in part, from quantitation of the samples against the buffered saline standard curves rather than plasma.

PCA extraction.—The carbohydrate-rich proteins (including CEA), which make up the seromucoid of serum and plasma, can be separated from most proteins and carbohydrate-poor proteins by PCA extraction (*23*, *24*). Pusztaszeri and Mach (*25*) observed a definite inhibitory effect when an excess of PCA extract of normal plasma (containing no added CEA) was tested in the Farr assay. Since excess amounts of PCA-*soluble* glycoproteins (far higher than found in "normal" sera or plasma) can interfere in the radioimmunoassay, possibly PCA-*precipitable* proteins and glycoproteins add to this effect. This may occur when normal amounts of serum or plasma are assayed without prior PCA extraction.

The use of PCA as a preliminary step in CEA tests presents several problems: *a*) PCA is an explosive reagent; *b*) native circulating CEA or CEA in tumor tissue may be altered by PCA; and *c*) PCA extraction necessitates dialysis and thereby lengthens the time requirement for any "indirect" assay system. Although many investigators wish to avoid these problems by eliminating the PCA step, they must ensure the specificity of their assay (i.e., the ability to distinguish CEA from immunologically cross-reacting antigens and molecules which simply interfere).

Preparation of CEA

The lack of rigorous standardization of antigens and antisera used in the different assays may cause variable results. The purification procedure used for the isolation of CEA has changed with time. First described by Krupey et al. in 1968 (*4*), it entailed PCA extraction, paper block electrophoresis, and Sephadex G-200 column chromatography; the antigen used in the assay by Lange et al. (*13*) (table 1) was so prepared. However, the antigen used in the initial report of clinical studies from Montreal in 1969 (*5*) was isolated by PCA extraction, Sepharose 4B and Sephadex G-200 gel filtration, and finally Sephadex G-25 block electrophoresis [as later reported by Krupey et al. in 1972 (*26*)]; antibody used in these studies was raised against this antigen (*5*). When the physicochemical analysis (table 2) of CEA prepared by Krupey et al. using the original procedure (col. 1) (*4*) is compared with that isolated by the improved procedure (col. 2) (*27*), differences (italicized in table 2) of ≥50% can be noted in concentrations of aspartic acid, leucine, isoleucine, tyrosine, lysine, histidine, fucose, mannose, galactose, and sialic acid. Although immunochemical identity of the CEAs prepared by the 2 procedures was indicated by radioimmunoassay and double immunodiffusion (*26*), the antisera used may have identified common antigenic sites rather than identical molecules.

The CEA used in the Z-Gel and Go assays was isolated by PCA extraction and diethylaminoethyl-carboxymethyl cellulose and Sepharose 6B column chromatography. Although this procedure provides CEA which has immunochemical identity with the CEA isolated by the improved procedure in Krupey's laboratory (table 2—col. 2) and similar physicochemi-

TABLE 2.—*Comparison of amino acid and monosaccharide composition of various CEAs*

	μmoles amino acid/mg CEA*			
	1	2	3	4
Amino acid:				
Aspartic acid	*0. 25*	0. 38	0. 44	0. 31
Threonine	0. 18	0. 22	0. 26	0. 18
Serine	0. 39†	0. 27	0. 30	0. 25
Glutamic acid	0. 25	0. 26	0. 31	0. 21
Proline	0. 20	0. 19	*0. 34*	*0. 10*
Glycine	0. 18	0. 13	0. 16	0. 14
Alanine	0. 12	0. 14	0. 18	0. 11
Leucine	*0. 04*	0. 22	0. 27	0. 18
Isoleucine	*0. 02*	0. 12	0. 15	0. 11
Tyrosine	*0. 04*	0. 06	*0. 12*	0. 08
Phenylalanine	0. 03	0. 05	*0. 09*	0. 04
Lysine	*0. 04*	0. 06	*0. 09*	0. 05
Histidine	*0. 07*	0. 03	*0. 06*	0. 03
Arginine	*0. 07*	0. 08	0. 11	0. 06
Valine		0. 16	0. 21	0. 14
Monosaccharide:	‡			
L-Fucose	*0. 87*	0. 15		0. 20
D-Mannose	*0. 31*	0. 49		*0. 33*
D-Galactose	*0. 93*	0. 52		*0. 26*
Sialic acid	*0. 13*	0. 24		*0. 07*
N-Acetyl-D-glucosamine		0. 97	0. 67§	*0. 62*
N-Acetylgalactosamine	N.S.‖	N.S.‖	0. 05§	N.S.‖

*1—Krupey et al. (*4*); values represent average for determinations on antigen from hepatic metastases from adenocarcinoma of rectum and of colon and a pool of 7 primary colonic adenocarcinomas. 2—Banjo et al. (*27*); antigen from hepatic metastases from a colon adenocarcinoma. 3—Terry et al. (*30*); values represent mean of determinations on antigen from hepatic metastases from colon adenocarcinoma in 2 patients. 4—Hansen (*32*).

†Values were not corrected for losses during hydrolysis.

‡Values represent mean of determinations on antigen from 1 hepatic metastatic tumor of the rectum and 1 hepatic metastatic tumor of colon.

§Coligan et al. (*31*).

‖ Not significant.

cal characteristics (table 2—col. 4), it may have an ion-sensitive site that is preferentially recognized in the Z-Gel assay (*7*).

The CEA used by Laurence et al. (*10*) in the modified double antibody assay was prepared in Todd's laboratory (*28*) according to the later method of Krupey et al. (*26*); but the phosphate-buffered saline used for elution off the Sepharose 4B and Sephadex G-200 columns was at pH 5.5 instead of at pH 4.5, and block electrophoresis was not used. (Laurence now uses CEA prepared by Krupey et al. and has found no changes in the standard curve or "normal levels.") Krupey found that block electrophoresis removed from CEA preparations a pigmented material which possessed an alpha mobility and which may account for some disparity in amino acid results (*29*). The CEA originally used by Laurence et al. was immunochemically identical and physicochemically similar to that of Krupey et al. (table 2—col. 3 compared to 2). When Terry et al. (*30*) analyzed the amino acid composition, the concentrations of proline, tyrosine, phenylalanine, lysine, and histidine differed by $\geq 50\%$ from those reported in column 2. Monosaccharide analysis of CEA prepared by the same investigators using a different procedure (*31*) revealed the presence of *N*-acetylgalactosamine, which was reduced to trace amounts by further purification with isoelectric focusing. Krupey and Hansen found trace amounts of *N*-acetylgalactosamine in some of their CEA preparations.

The differences noted in the monosaccharide composition of the various CEAs may be the most significant. Banjo et al. (*27*) suggested that a relatively high concentration of *N*-acetylglucosamine is of major importance in the immunologic determinant site of CEA. *N*-acetylglucosamine was not identified as a major constituent of the original CEA preparations, due to insufficient material for adequate analysis (*see* table 2). The variations in sialic acid concentration, as identified by Banjo et al. (*27*) and Coligan et al. (*31*), may contribute to differences in electrophoretic mobility but not necessarily to variations in the immunologic characteristics of different CEA preparations (*32*). Some of the dissimilarities noted in table 2 may be due to *a*) purification techniques, *b*) tissue sources, *c*) criteria of purity, and *d*) improved methods of analysis.

Despite the chemical heterogeneity described for CEA from tissues of different individuals, antigenic identity of the CEA preparations was seen (*31, 33*). Additional information is required to distinguish the heterogeneity of CEA from impurities that may be associated with CEA due to isolation procedures. Since all test values are based on comparisons with the standard values obtained in the respective assays, the purity and exact quantitation of CEA preparations used are critical. Before use in standard curves, CEA (of relatively low purity) must be assayed for "activity" (purity). Furthermore, radioactive labeling of impure CEA for use in the test may result in erroneous results, if the anti-CEA antiserum used in the assay or non-CEA antibodies in the test specimen recognize the impurities immunologically. Impure CEA for immunization of animals may result in nonspecific antisera requiring extensive absorption.

Immunologically and physicochemically characterized international "standards" of CEA are necessary, which can be assayed in individual laboratories to relate findings obtained by different tests. Until such standards are available, CEA can only be defined, as suggested by Martin and Devant (*34*), as an antigen giving reaction of complete identity with the immunologic system prepared in Gold's laboratory. This is difficult to attain with certainty, as none of the currently available immunologic methods offer complete proof of identity. The combined results of many procedures, such as immunodiffusion, radioimmunoassay, absorption, and immunoelectrophoresis, are necessary to define CEA *immunologically*.

Preparation of Anti-CEA Antisera

A variety of preparations of CEA and several differing procedures have been used to immunize animals to raise anti-CEA antisera. To obtain antisera of high titer and affinity which required absorption, Gold and Freedman (*1, 2*) and Hansen et al. (*35*) used multiple injections of milligram quantities of purified CEA mixed with Freund complete adjuvant in rabbits and goats. To obtain high-affinity antisera and minimize formation of antibodies directed against non-CEA contaminants so that absorption was unnecessary, Egan et al. (*9*) immunized goats with microgram quantities of purified CEA in Freund complete adjuvant. To minimize the need for absorption, Saravis et al. (*36*) immunized rabbits, previously made tolerant to normal tissue antigens, with microgram quantities of semipurified CEA in aqueous aluminum hydroxide gel. Mach and Pusztaszeri (*37*) immunized rabbits with milligram quantities of semipurified CEA and absorbed the resulting antisera with PCA extracts of normal gut, lung, and plasma. When the absorbed antisera gave single precipitin lines with CEA by double diffusion in agar, the washed precipitate was used with Freund adjuvant to immunize another series of rabbits. Burtin et al. (*38*) immunized rabbits with milligram quantities of PCA extracts of colon cancer in Freund adjuvant and other rabbits with CEA prepared by 2 successive gel filtrations of PCA extracts on Sephadex G-200. They then absorbed these antisera with *a*) normal human plasma, *b*) saline and PCA extracts of normal digestive mucosa, and *c*) A and B red blood cell stromas. Martin and Martin (*39*) immunized rabbits with microgram quantities of purified CEA in Freund complete adjuvant and absorbed the resulting antisera with normal human plasma and PCA extracts of normal spleen, stomach, and colon.

Each antiserum prepared above was compared, either directly or indirectly, in Ouchterlony immunodiffusion against anti-CEA antibody obtained from Montreal [which was raised against CEA prepared by the 1972 method of Krupey et al. (*26*)], and lines of identity were observed. This did not prove the antisera were identical but rather that they recognized common site(s). Indeed, several authors showed that their own anti-CEA antisera could identify antigens similar but not identical to CEA (*see* below). Whether the positive findings obtained on sera and plasmas of patients with nonmalignant diseases are due to CEA (as characterized by Krupey et al.) or to the presence of such sites on CEA-like antigens is not yet clear.

Identification of CEA-Like Antigens

Several investigators identified CEA or "CEA-like" antigens in normal digestive tract as well as in malignant and normal nondigestive tract tissues (table 3). *a*) Martin et al. (*40, 41*) "absorbed out" an anti-CEA antiserum with high concentrations (80–160 mg/ml) of PCA extracts of noncancerous colonic mucosa and thereby demonstrated the presence of antigen in the extracts. *b*) von Kleist and Burtin (*42*) showed the presence of CEA-active material in normal colonic mucosa with immunofluorescent anti-CEA antiserum. Burtin et al. (*38, 43*) subsequently identified CEA activity in benign polyps, juvenile colonic mucosa, and hemorrhoidal mucosa. *c*) LoGerfo and Herter (*44*) identified CEA activity in normal colonic mucosa and lung. *d*) By reacting partially purified tissue extracts (PCA extraction and Sephadex G-200 column chromatography) against anti-CEA antibody in Ouchterlony gel diffusion, Pusztaszeri and Mach (*25*) found CEA activity in normal lung, lactating breast, and carcinoma of the lung and breast. *e*) We extracted from normal and cirrhotic liver, antigen(s) showing immunochemical identity to CEA by radioimmunoassay, Ouchterlony, and precipitin inhibition techniques (*45*).

We identified in normal human serum an antigen with CEA activity in a concentration of 0.5–1 ng/ml, and Chu et al. (*46*) found similar concentrations in normal plasma. The relatively low levels of CEA in the serum and plasma of normal individuals appear to be consistent with the finding of CEA (or an antigen immunochemically identical to it) in low concentrations in normal digestive tract mucosa. The reasons for the mildly elevated values in some patients with nonmalignant diseases are not yet known. In alcoholic cirrhosis (*18*), the damaged liver may fail to metabolize normally low levels of circulating antigen. In ulcerative colitis (*19*), increased amounts of antigen may be produced in or otherwise released into the circulation by the inflamed tissue.

CEA activity has also been demonstrated in human feces (*47, 48*), urine (*48, 49*), meconium (*50*), and

TABLE 3.—*Relative CEA concentrations in normal and malignant tissues*

Investigators	Tissue	Approximate ratio CEA	
		Normal/ corresponding tumor	Normal/ colonic cancer
Martin et al. (*40, 41*)	Normal colon	N.D.*	1/40
	Normal liver	N.D.	1/1000
Burtin et al., von Kleist and Burtin, Burtin et al. (*38, 42, 43*)†.	Normal colon	N.D.	N.D.
	Polyps	N.D.	N.D.
	Juvenile colon	N.D.	N.D.
	Hemorrhoidal mucosa	N.D.	N.D.
LoGerfo and Herter (*44*).	Normal lung	N.D.	1/400
	Normal colon	N.D.	1/20
Pusztaszeri and Mach (*25*).	Normal lactating breast	1/5	1/240
	Normal lung	1/25	1/2000
Kupchik and Zamcheck (*45*).	Normal liver	—	1/10,000
	Cirrhotic liver	—	1/5000

*N.D.=the tissue was studied but quantities were not determined.

†The intensity of fluorescence was less in the normal tissues when compared against malignant.

saliva (*34*). However, the sources of the activities demonstrated have not been identified nor have the substances been fully characterized.

Several laboratories identified antigens immunochemically similar to CEA. Designated as "colon carcinoma antigen-III" (CCA-III) (*51*), "non-specific cross-reacting antigen" (NCA) (*52*), "normal glycoprotein" (NGP) (*37*), or "colonic carcinoembryonic antigen-2" (CCEA-2) (*53*), these antigens may all prove to be the same molecule. They showed immunochemical identity to each other on Ouchterlony analysis, were of approximately the same molecular weight (50,000), and were PCA-soluble glycoproteins. They showed only partial identity with CEA. To date, none has been shown to influence radioimmunoassays for CEA. NGP and CCA-III were used to absorb out anti-CEA antisera and did not affect CEA levels obtained by radioimmunoassay on patients' plasma (*25*, *32*). NCA did not react in the radioimmunoassay used by Franchimont et al. (*54*) nor did CCEA-II react in the modified double antibody test used by Laurence (*55*).

Human Anti-CEA Antibody

Evidence for the presence of autoantibodies.—In 1967, using the bis-diazotized benzidine-hemagglutination technique, Gold (*56*) described a circulating antibody to CEA in 30 of 43 patients with nonmetastatic colonic carcinoma but in none of 30 such patients with metastases nor in 14 normal individuals. In addition, IgM anti-CEA antibodies were found in the sera of 17 (57%) of 30 pregnant women and 2 of 39 patients with noncancerous disease of the digestive system. The finding of Dyce and Haverback (*57*), that CEA was bound by a substance in a gastric adenocarcinoma, may be supportive evidence for the presence of anti-CEA antibodies. Collatz et al. (*58*) using 3 different techniques (passive hemagglutination, immunoabsorption, and immunofluorescence) could not find anti-CEA antibody in the sera of any of 190 patients (including 125 with digestive tract cancer). However, they did find antibodies to normal colonic proteins. LoGerfo et al. (*59*), using radiolabeled CEA as a marker and molecular filtration, column chromatography, or Z-Gel precipitation, could not identify circulating antigen-antibody complexes. The inability of these workers to verify the presence of anti-CEA antibodies in the serum of patients with carcinoma of the colon prompted Gold and his co-workers to develop a sensitive primary binding technique for the detection of antibody (*60*). Gold et al. (*60*), using a radioimmunoelectrophoresis technique, detected anti-CEA antibodies in 9 of 19 patients with gastrointestinal cancer. They also demonstrated cross-reactivity between blood group A substance and CEA (*see* below). If CEA is an antigen found in normal tissues, anti-CEA antibodies in the serum of cancer patients may be explained by a mechanism analogous to that operating in autoimmune diseases. An alternative possibility is that the antibody is polyvalent and cross-reacts with several antigens or antigenic sites.

Effect of radioimmunoassay.—Possibly the low occurrence of circulating CEA positivity in patients with early primary colonic carcinoma (*61*) is not due to small amounts of CEA, but rather to the presence of circulating anti-CEA antibody. The presence of an antigen-antibody complex would limit the identification of CEA in the radioimmunoassays, as Dyce and Haverback suggested in the instance of tissue obtained from a gastric carcinoma (*57*). When Martin et al. (*62*) precipitated CEA with rabbit anti-CEA antibody and then extracted with PCA, they recovered only 40% of the added CEA, compared to 90% recovered when CEA was extracted with PCA before binding with antibody. They suggested that the lower recovery was due mostly to nonreversible absorption of CEA on the immunoglobulins, after precipitation by PCA. We did not find measurable quantities of CEA in the PCA-insoluble precipitates of CEA-negative plasmas obtained from patients with colonic carcinoma (*63*).

In direct assays (PCA not used), human anti-CEA antibodies could be manifest in several ways. When $AmSO_4$ or Z-Gel was used to coprecipitate antibodies and ^{125}I-CEA bound to antibody, CEA could bind the human antibody and not inhibit the binding of ^{125}I-CEA to goat anti-CEA antibody. This would decrease the amount of antigen detected by the radioimmunoassay. On the other hand, in the double antibody assay, in which a second antibody was used to precipitate only the goat anti-CEA antibody, at least some ^{125}I-CEA (depending on the amount of human antibody present) would be bound by the human antibody. This would be reflected by a decrease in the precipitable ^{125}I-CEA and would appear to represent increased CEA activity.

Further studies are required to settle the question of the presence or absence of circulating antibodies to CEA as well as the relationship of CEA to blood group antigens. If sensitive tests can be developed to identify anti-CEA antibodies and distinguish them from anti-blood group A antibodies (*see* below), they would provide an important adjunct to the CEA assays for early diagnosis of cancer.

Relationships of CEA to Blood Group Antigens

Stillman and Zamcheck (*64*) first questioned the relationship between CEA and blood group glycoproteins. Davidsohn et al. (*65*, *66*) and then Sheahan et al. (*67*) demonstrated the loss of tissue blood-group specificity in human cancerous tissues.

The observation by Hansen et al. (*7*), that some CEA preparations may be contaminated by human blood group A substance, was confirmed by LoGerfo et al. (*59*) and Turner et al. (*68*). These findings brought concern that the rabbit or goat antisera raised against such preparations in radioimmunoassays would increase the number of "false" positives. In fact, Turner et al. (*68*) reported that one preparation of goat anti-CEA antiserum from Montreal contained anti-A antibodies. However, this laboratory, using a different goat antiserum obtained from Montreal, and Reynoso et al. (*21*), using goat antiserum raised against CEA isolated by Hansen, found no apparent correlation between patient blood groups and CEA levels. Gold et al. (*60*) and Turner et al. (*68*) identified blood group A activity in CEA which they did not consider a contaminant. Anti-blood group A antibodies bound CEA, and this effect could be abrogated by prior absorption of the antibody with blood group A red cells or A substance. In addition, Gold reported that human anti-CEA antibody also cross-reacted with blood group A substance. These findings were not compatible with the observations of Krupey et al. (*4*), Banjo et al. (*27*), and Hansen (*32*) (*see* "Preparation of CEA") who found little or no *N*-acetylgalactosamine (the immunoreactive blood group A monosaccharide in CEA). Coligan et al. (*31*) did find *N*-acetylgalactosamine in some CEA preparations, of which only traces remained after further purification of the CEA by isoelectric focusing.

Mach and Holburn (*69*) recently showed blood group A, B, and Lewis a and b activity intimately associated with some CEA preparations which were immunologically identical to CEA preparations from Montreal when tested against Montreal goat anti-CEA antibody. They suggested that labeled CEA containing blood group antigen activity could influence the results obtained when used in direct radioimmunoassays for CEA, since antibodies to the blood group antigens are usually present in the test specimens. Recently, Simmons and Perlmann (*70*), studying the relationship between CEA and blood group antigens, suggested that CEA extracted from entodermally derived adenocarcinomas represents incomplete blood group substances of the ABO system. However, Gold (*71*) found only 4% CEA activity (on a dry wt basis) and "different physicochemical properties" in the antigen preparation studied by Simmons and Perlmann.

Investigators should try to ensure that the CEA and anti-CEA preparations used in their radioimmunoassays do not manifest blood group antigen or antibody activity.

Comment and Summary

The initial hope for a simple and clinically practical specific test for cancer of the colon has not yet been realized. Increasing is the awareness of the complexity of the immunologic problem and the diversity of methodologies. The number of publications concerning the immunochemistry of CEA and assays for its detection is multiplying rapidly. The physician who proposes to use these methods for clinical purposes of diagnosis, prognosis, or evaluation of therapy must be aware of the influence of methodology on the actual test findings. Hence it seemed desirable to summarize some of these considerations.

The original clinical investigation by the Montreal laboratory indicated a specificity of the Farr radioimmunoassay for gastrointestinal cancer. However, elevated levels of CEA have since been seen in nongastrointestinal malignancies and some nonmalignant diseases by various investigators (including the Montreal group) using different CEA and anti-CEA antibody preparations and different techniques. Despite the different systems used, the findings in patients have been similar. The Farr assay requires the largest volume of blood and the longest time to perform; we agree with Freedman (*72*) that, due to its technical complexity, the Farr assay is likely to be replaced by simpler methods. Indeed, investigators should be encouraged to continue to evaluate such procedures.

Several tests for the detection of CEA in blood are available. They are influenced by numerous factors: *a*) Non-CEA or CEA-like substances may affect the assays either by immunologic cross-reaction or by nonspecific inhibition of the immunologic reaction; *b*) immunochemical characteristics of the antigens and antisera used depend on the preparative procedures; *c*) circulating anti-CEA antibodies (if present) may bind or otherwise obscure the presence of circulating CEA; *d*) contamination or cross-reaction with blood group antigens may influence results obtained; and *e*) the definition of "normal" varies with different methods and in different laboratories. These and other factors which influence the results of any given method used as an adjunct to the clinical diagnosis and management of cancer patients must be appreciated if reliable performance and safe and useful interpretation are to be expected.

When antigens and antisera are standardized in the various assays, each may be useful for clinical application as long as the clinical use and limitations of the test have been carefully defined.

Much work under way promises to clarify some of the problems and to lead to improvement and standardization of methodology.

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ADDENDUM

During the past 5 years, CEA has been the subject of numerous investigations. The following represent only a small portion of relevant publications [*see (1, 2)* for review].

In an effort to identify and isolate species of CEA with antigenic determinants more specific for tumor cells, investigators used various isolation procedures (*3–7*). Further studies are necessary to determine whether antisera to forms of CEA such as CEA-S (*8*) or CEA-M (*9*) can be used in clinical radioimmunoassays to provide the cancer specificity lacking to date for CEA.

To permit earlier and more accurate diagnosis of cancer, many investigators have assayed for CEA in body fluids (*10*) including those of the pancreas (*11, 12*), stomach (*13–15*), and duodenum (*15–18*), bile (*19*), and ascites (*20*), pleural (*21*), and spinal fluids (*22*). In general, these studies indicated some quantitative correlation between the level of CEA activity in each of the tissues or fluids and the presence or absence of neoplasms.

Goldenberg et al. (*23*) have recently shown that radiolabeled antibody to CEA can be used successfully for the detection and localization of cancers by external photoscanning. Refinement of such a procedure would present an additional clinically useful aspect of CEA.

Studies from this laboratory further emphasized the role of the liver in the regulation of circulating CEA levels (*24–26*). The liver appears to have a limited ability to metabolize and/or excrete the potentially large load of CEA produced by colon cancer. Benign or malignant disease can further impair liver function by replacing liver tissue with tumor, by obstructing bile outflow, or by other means.

Most clinical studies during the past 5 years have confirmed the usefulness of serial CEA levels in the management of patients with cancer (*27–30*) and as a monitor of chemotherapy (*31–38*) or radiation therapy (*34*). Its potential as an indicator for second-look surgery is under study (*35, 36*). For such use, the assay must be reproducible. Martin et al. (*36*) proposed the use of a "nomogram" to determine patient-related changes in low levels (<10 ng/ml). Studies by this laboratory (*38*) and others [Rule and associates (*39*), Madsen et al. (*40*), and Taylor and his co-workers (*41*)] showed that a disparity of values occurs when shifting from the indirect (for values <20 ng/ml) to the direct (for values >20 ng/ml) Z-gel assay. Thus when a patient is being followed by serial determinations, a change from 19 to 50 ng/ml or the reverse may not be clinically significant, and caution is advised in interpreting such levels. Clinicians need to bear in mind that methodological differences in technical performance of the CEA assay in different laboratories may also influence the values reported.

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Note:

Volume 42, 1978, of *Cancer* is devoted to the International Conference on the Clinical Uses of CEA (Lexington, Ky., June 1–4, 1977) and provides an update of CEA studies.

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In Vitro Assays of Cell-Mediated Immunity to Human Solid Tumors: Problems of Quantitation, Specificity, and Interpretation [1]

The concept that immunologic factors have a function in control or even prevention of neoplastic disease has developed following well-substantiated investigations showing that many, although by no means all, experimental animal tumors, including those of unknown (spontaneous) etiology, express neoantigens capable of eliciting immune rejection reactions comparable in kind, if not in degree, with those causing rejection of normal allografts (*1–3*). Cell-mediated immunity is generally considered primarily responsible for tumor rejection in these experimental tumor systems, since resistance can be transferred to normal compatible hosts by means of lymphoid cells from tumor-immune or even tumor-bearing hosts (*4*). Although this opinion may have to be modified because of recent developments indicating antibody-mediated killing through complement- or cell-dependent mechanisms (*2*), it is apparent that in vitro monitoring of cell-mediated immunity provides an approach for evaluating the role of immunity in human neoplastic disease. Of the many procedures developed, the colony-inhibition technique (*5*) has been considered sufficiently sensitive to allow measurement of the action of sensitized lymphoid cells on tumor cells in culture. This has been modified subsequently as a microcytotoxicity assay in which survival of tumor cells plated into the wells of plastic microtest dishes and exposed to sensitized lymphoid cells, e.g., taken from tumor-bearing patients, is compared with that of controls treated with lymphoid cells from either normal individuals or patients with nonneoplastic disease (*3, 6*). The original justification for use of these tests was provided by experimental animal studies in which it was possible to demonstrate cell-mediated immune reactions with specificities identical to those evaluated in vivo by rejection of tumor transplanted into syngeneic hosts. For example, lymph node cells from hosts bearing Moloney sarcoma virus-induced tumor were cytotoxic for Moloney sarcoma cells but not for other virus-induced tumors (*7*). By use of a range of aminoazo dye-induced rat hepatomas, each of which expresses individual tumor rejection antigens, it could be shown also that in vitro lymphocytotoxicity assays detected neoantigens with similar specificities (*8*).

Following the experimental animal studies, the microcytotoxicity assay, involving visual counting of target cells and variants by use of radioisotope-labeled tumor cells, has been used to evaluate the cytotoxicity of peripheral blood lymphocytes from cancer patients (*3*). In many original studies it was reported that tumor-specific responses were detected, since blood lymphocytes were cytotoxic for cells derived from the patient's tumor but not for cultured normal cells. Lymphocytes from controls, including normal subjects and patients with nonneoplastic disease, were likewise not cytotoxic for the tumor cells. The specificities of the tumor antigens thus detected with human tumors, however, differed from those of experimental animal tumors in that they were related to the histologic type of the tumor. For example, blood lymphocytes from a patient with colon carcinoma were cytotoxic for the patient's own tumor and for other colon carcinomas but not for histologically different tumors, e.g., bladder carcinoma and melanoma (*9, 10*). Based on these original studies, the microcytotoxicity assay has been used in a wide range of human tumor studies, especially with a view toward establishing whether circulating humoral factors have a function in the tumor-host relationship (*3*). Also, the test is being used to monitor tumor-immune responses in patients undergoing therapy (*11, 12*). This has led, however, to a considerable divergence of opinion about the tumor specificity of the cell-mediated reactions detected in this test, and a stage has been reached where a critical appraisal of the technology of the assay is required giving attention to *a*) selection of target tumor cells, *b*) characterization of tumor-associated antigens, *c*) preparation and characterization of effector cells, and *d*) standardization of the microcytotoxicity assay.

Target Tumor Cells

The choice at present is between short-term cultures established from individual patients and the use of continuous cell lines. Most early studies in which lymphocytotoxic reactions showed tumor specificity employed short-term cultures, and the value of continuous cell lines remains to be established. For example, in one melanoma study, lymphocytes from (selected) melanoma patients were cytotoxic in 10 of 11 tests compared with

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[1] Supported by grants from the Cancer Research Campaign.

Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

2 of 12 normal donor preparations when tested on seven short-term melanoma cultures (*13*). In comparison, all lymphocyte preparations were cytotoxic in tests on melanoma cells derived from three established lines. Similar discrepancies have been obtained in tests on mammary carcinoma cells in culture (Della Porta G: Personal communication), and studies of this type suggest that different groups of neoantigens may be detected on short-term and established cell lines. This presents a dilemma, since the use of short-term lines is restricted by the success rate in establishing such cultures. Even more important, the use of short-term cultures limits the effectiveness of collaborative studies between different laboratories, this now being a necessary development for standardization of the microcytotoxicity assay.

Characterization of Tumor-Associated Antigens

The above comments indicate that attention must be given to characterization of the tumor-associated antigens involved in the in vitro cytotoxic reactions produced by peripheral blood lymphocytes from cancer patients. In this context, in vitro cell-mediated cytotoxic reactions do not *necessarily* reflect in vivo tumor rejection responses. This is illustrated by studies on spontaneously arising rat mammary carcinomas and sarcomas lacking the capacity to induce significant immunity against tumor cells transplanted into syngeneic hosts; yet lymph node cells from tumor-bearing rats are cytotoxic in vitro for appropriate target cells (*14*). The neoantigens detected on these tumors, showing organ type specificity and therefore similar to those identified on human tumors, have been identified as reexpressed fetal antigens, since tumor-bearer lymph node cell cytotoxicity was blocked by preexposure of target cells to serum from multiparous animals. Cross-reacting fetal antigens have been identified also on rat colon carcinomas (*15*) and, in comparison, blood lymphocytes from colon carcinoma patients were shown to be cytotoxic for both tumor and fetal gut cells (*9*).

These comparative studies suggest that the organ type-specific neoantigens associated with human tumors may be fetal antigens. This possibility could be resolved by use of serum from multiparous humans to block tumor cells from attack by blood lymphocytes from cancer patients, providing that responses to HL-A antigens were controlled. Alternatively, neoantigens on human tumors could be characterized by assay of the capacity of isolated tissue antigens, e.g., from solid tumor or embryo tissues, to specifically inhibit the in vitro cytotoxicity of human peripheral blood lymphocytes. This approach has been utilized with a number of experimental animal tumors where it has been possible to characterize tumor-specific antigens and also to differentiate these from tumor-associated embryonic components (*16–20*). In comparison, tests with several human tumors, including melanoma and colon carcinoma, indicate that tumor antigen isolated from solid tissue specifically inhibits lymphocyte cytotoxicity for appropriate target tumor cells (*21, 22*) though the characteristics of the neoantigens have yet to be defined.

Preparation and Characterization of Effector Cells

Interpretation of in vitro lymphocytotoxic reactions against human tumors is further complicated by the lack of knowledge about the effector cells(s) involved. In urinary bladder tumors, for example, non-T cell responses have been implicated, since cytotoxicity was lost when this population of lymphoid cells was removed, e.g., by passage through glass bead columns coated with human immunoglobulin–rabbit antiserum to human immunoglobulin or by rosette formation (*23, 24*). In contrast, T cell-enriched subpopulations isolated by spontaneous rosette formation with sheep erythrocytes have been shown to be more cytotoxic than T cell-depleted fractions of peripheral blood lymphocytes from patients with melanoma (*25*) or colon carcinoma (*22*). However, in another melanoma study, cytotoxic reactivity again was found to be associated with the non-T (non-erythrocyte-rosetting) population of blood lymphocytes (*13*). These contradictory findings imply that more than one type of effector cell (e.g., T cell and antibody-dependent non-T cell) may be involved in recognition of the same (or different) neoantigens on human tumor cells. Also, as already demonstrated with the Moloney sarcoma virus-induced tumors, the relative contribution of different effector cell mechanisms may change with the stage of the disease (*26–28*). These examples illustrate why inconsistent data may be produced by assays employing lymphocyte populations separated by relatively unsophisticated techniques. Many studies so far published have used one of two procedures to separate lymphocyte-enriched populations, i.e., sedimentation under unit gravity through a dense medium such as plasmagel or centrifugation on a Ficoll-Hypaque density gradient. Neither of these procedures is completely adequate and a more critical appraisal should be made of the cell populations used for microcytotoxicity tests. Of particular importance may be the degree of contamination of lymphocyte preparations with monocytes, since this subpopulation could contribute significantly to "non-specific" target cell killing (*29*). It is also necessary to take into account the actual recoveries of cells in enriched populations, because lymphocytes are lost during separation. It has generally been assumed that such losses occur randomly, but possibly cells are lost in a more selective manner, and this may be important in view of evidence for T-cell populations with different functions including regulator or suppressor activity (*28, 30, 31*).

Standardization of Microcytotoxicity Assay

It is now generally accepted that the microcytotoxicity assay, involving prolonged cocultivation of tumor and lymphoid cells, detects a number of in vitro cellular events, including activation and maturation of effector cells, that may be cytostatic or cytotoxic for the target tumor cell. Although it might be argued that this test closely reflects in vivo conditions, clearly such a multiplicity of responses against an array of tumor-associated products may not result in a demonstrable specific response. For example, cultivation of peripheral blood lymphocytes from sarcoma patients with autologous tumor cells leads to the generation of "non-specific" killer cells, these being cytotoxic for both sarcoma cells and normal fibroblasts (*32*). Sensitization of patients' lymphocytes against human melanoma-associated antigens to yield specifically cytotoxic cells has also been reported (*33*), but in other studies both specific and non-specific responses were observed (*34*).

Since short-term cytotoxicity assays, e.g., ^{51}Cr-release, are not yet applicable to most types of human solid tumor, attention should be given to more rigorous standardization of the microcytotoxicity test. Of particular importance in this respect is the choice of controls, because in many instances comparison of the survival of tumor cells after treatment with a patient's or normal subject's blood lymphocytes cannot be related simply to survival in "media-only" controls. Normal lymphocytes may sometimes exert a "feeder effect," and corrections for this by application of approximations calculated from titration curves are not entirely satisfactory (*35*). Also, in some instances lymphocytes from normal individuals may be even more cytotoxic than patients' lymphocytes for target tumor cells, especially when established cell lines are used (*13*). This may reflect cytotoxic responses against antigens associated with tumors that are irrelevant to the neoplastic process, e.g., tissue-specific antigens, and this hypothesis is open to experimental verification by incorporation of blocking or inhibition assays with appropriate reagents such as tissue-specific antibody or tissue antigen extracts. Alternatively, normal lymphocytes may have a greater capacity than those from cancer patients to respond to tumor-associated antigens during the prolonged incubation period of the microcytotoxicity test. These problems are emphasized by recent population studies (*35*) in which simultaneous assays of blood lymphocytes from normal individuals and patients with different types of neoplastic disease, on a range of target tumor cells, failed to demonstrate any tumor specificity in the responses. In view of the variable and unpredictable effects of normal lymphocytes, it is clear that cytotoxic responses must eventually be related to controls by use of target tumor cells without any added effector cells. This may require the inclusion of controls to differentiate between cytotoxic responses triggered by tumor-specific and other irrelevant antigens expressed on the tumor cells.

Conclusion

Discordant findings are being reported from different laboratories on the cytotoxicity of peripheral blood lymphocytes for cultured human tumor cells, and these differences must be resolved if the test is to be generally used for evaluation of cell-mediated immunity in human neoplastic disease. This may happen through cooperative studies in which reagents such as target tumor cells are exchanged, though a more effective approach would be to arrange exchange visits among laboratory personnel. It may be questioned also whether the procedures in many laboratories are as rigorously standardized as is desirable, e.g., in defining the composition of "purified" lymphocyte preparations, and an important step would be the publication of acceptable protocols.

Even with reservations about the standardization of the microcytotoxicity assay, there are sufficient published reports to show that under "laboratory-defined" conditions the test can yield meaningful data, e.g., in characterizing humoral factors interfering with cell-mediated immunity in the tumor-bearing host (*3*). Furthermore, correlations of changes in serum blocking or inhibitory activities and clinical response in patients receiving immunotherapy suggest that these tests may have prognostic significance (*12*). In a consideration of the clinical relevance of these studies, it is pertinent that the findings are generally in accord with the now substantial evidence derived from well-controlled animal tumor systems showing that humoral factors in the tumor-bearing host can abrogate tumor-specific, cell-mediated immunity. Also, comparable findings on cell-mediated immunity to human neoplasms and its abrogation by serum factors have been obtained by other in vitro techniques (such as leukocyte migration inhibition and leukocyte adherence inhibition) to monitor tumor-immune reactions (*36–39*). Most of these techniques employ a secondary reagent, such as tumor-bearer serum or isolated tumor antigen, to modify in vitro effects; as already noted, inhibition of effector cell cytotoxicity by tumor antigen may be the best way to incorporate specificity controls in the microcytotoxicity assay.

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ADDENDUM

Following the criticisms raised by the above and the associated Editorial (*1*), the problems of in vitro tumor cell killing by lymphocytes from normal individuals received more serious consideration. This led to the more general acceptance that lymphoid cells from nonsensitized individuals may be cytotoxic for neoplastic cells and the phenomenon of natural cell-mediated cytotoxicity is now well established (*2*). Indeed such has been the growth in this area of research that many investigators consider this to be a major defense mechanism against neoplasia (*3, 4*). Furthermore, the finding that natural killer cells can be augmented by immunomodulators such as BCG and *Corynebacterium parvum* (*4, 5*) indicates that these effector cells may be important in immunotherapy (*6*). This concept needs to be critically evaluated, since a number of clinical trials now include repeated administration of BCG in addition to chemotherapy and/or radiotherapy for the treatment of metastatic cancer (*6, 7*).

Since the criticisms of the in vitro assays of cell-mediated immunity to human solid tumor, little significant progress has been made in developing alternative methodologies. The problems of subjective errors and quantitation in the microcytotoxicity tests have largely been overcome by the development of assays that require radiolabeled target cells (*8*). However, none of these approaches have resolved the more fundamental problems arising after the lengthy in vitro incubation of lymphoid cells with tumor cells (*9*). In this respect, it is a little disconcerting to find that the assays currently in use, such as the leukocyte migration and adherence inhibition tests, are even less precise than the microcytotoxicity tests (*10–12*). In many instances, the principles of the assays have not been adequately substantiated, and, as with the cytotoxicity tests, discordant findings are being obtained by workers in different laboratories. One hopes that the lessons learned from the over-rapid development of microcytotoxicity tests in uncontrolled clinical studies will be heeded; thus the results of international workshops evaluating these tests are awaited.

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EDITORIAL

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Problems Associated With Study of Cell-Mediated Immunity to Human Tumors by Microcytotoxicity Assays

There is a considerable need for assays of cell-mediated immunity to human tumors, which could be used reliably to monitor the course of disease in cancer patients or for immunodiagnosis (i.e., to distinguish patients with cancer of a particular type from patients with other types of cancer, from patients with benign diseases, and from normal individuals). Several studies [e.g., (*1–7*)] initially suggested that the colony-inhibition or microcytotoxicity assays would be useful for both objectives. All these reports indicated that patients with cancer had significantly more cytotoxic reactivity than did normal individuals against tissue culture cells derived from tumors of the same histologic type (designated histologic type-specific reactivity) and not against cultures derived from normal cells or from tumors of other histologic types. Few or no normal controls were shown to have significant cytotoxic reactivity. Since then, the specificity of the observed reactions and the relative lack of normal reactivity in these assays have been increasingly questioned. In June 1972, in a conference held at the National Cancer Institute (NCI), some investigators presented data indicating frequent cytotoxic reactivity by normal individuals and a lack of complete histologic type-specific reactivity by cancer patients (*8–10*). Since then, many laboratory workers, including some of those initially obtaining good specificity, have produced similar patterns of results [e.g., (*11–14*); Fossati G, Canevari S, Della Porta G: Personal communication]. In a recent paper in this Journal, Takasugi et al. (*15*) described studies with large numbers of cancer patients and controls tested against a wide variety of cultured cell lines, and concluded that the specificity of cell-mediated cytotoxicity assays is not related to the histologic type of cancer.

What are the reasons for the wide range in the patterns of results? Two main possibilities need to be considered. 1) There are critical technical aspects of the microcytotoxicity assays that can significantly affect the results, and different researchers deal with these important technical variables in substantially disparate ways. 2) As stressed by Takasugi et al. (*15*), the differences seen among laboratory personnel are related to study design, and the problems with the results are due to fundamental aspects of the biology of the human tumor systems. There has been a series of attempts in the last few years to resolve these issues. Two workshops were held, one in 1971 (*16*) and one in 1974, during which investigators from different laboratories performed their assays with lymphocytes from the same donors and with the same target cells. At the 1972 NCI conference (*17*) and at a recent conference in London, sponsored by the International Union Against Cancer, representatives from many laboratories discussed at length the differences between the assays and the possible reasons for disparate results. In addition, there have been, and continue to be, some direct comparisons of various tests and technical variables between different laboratories and within the same laboratory. These efforts have identified a series of questions about technical details in the assays, about problems with base-line controls, and about other features in the design of experiments. Several of these questions are now being critically examined in some laboratories. This is an appropriate time to discuss the issues in some detail, and to try to place them in proper perspective.

Technical Problems Affecting Microcytotoxicity Assays

The technical features suggested to be important in the microcytotoxicity assays can be discussed in the context of the test systems themselves, the base line used for calculation of results, the tissue culture cells selected as the target cells and the preparation of the effector cells.

In regard to the assays performed by various researchers, it is important to consider first the objectivity of each test, the ability to quantitate results and particularly the reproducibility of results. The original colony-inhibition and microcytotoxicity assays depend on visual counting of large numbers of cells and are therefore tedious and susceptible to reader bias and other subjective factors. Therefore, many investigators have tried to make their assays more objective by use of electronic counting of target cells (*18*) or by labeling of target cells with radioisotopes (*8, 9, 19*). The initial studies were done with only one concentration of effector and target cells, and the results were dealt with qualitatively, as either significantly positive or not [e.g., (*2*)]. More recently, several workers have stressed quantitative aspects in their tests by using a range of effector to target cell ratios [e.g., (*10, 11, 20*)]. Such titrations led the Hellströms (*20*) to conclude that, contrary to their previous reports, patients with advanced melanoma were less reactive than patients with more localized or no evident disease.

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Editor's note: Periodically the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

Regardless of the method used to enumerate target cells remaining at the end of the assay or of the attacker to target cell ratios used, considerable differences among investigators exist because of variations in the calculation and expression of results. The calculation method and statistical manipulations used to determine significant differences can affect the end results, especially in the frequent cases when one is dealing with small numbers of remaining target cells at the end of the assay [*see* "Discussion" in (*17*)].

The reproducibility of results from day to day is an important issue when one tries to use these tests to monitor serially patients with cancer. However, scant attention has been paid to this problem. Heppner et al. (*12*) found considerable variation in the activity of the same normal donors tested repeatedly. We found good reproducibility when assays were run several days in a row with the same normal individual, but wide divergence when run at more widely spaced intervals (*8*).

One of the main factors making it difficult to assess clearly the reproducibility of the assays is the problem with identifying a suitable and stable base line for calculation of results. The initial studies, and most subsequent ones, used the results obtained with lymphocytes from a normal donor as the base-line control. Once one accepts the possibility for cytotoxic reactivity by some normal individuals (*see below*), this presents real difficulties in setting as zero the results of one unselected, normal donor. Some investigators have tried to avoid this by using the "media control," i.e., the target cells alone without added lymphocytes as the base line (*11, 12, 21, 22*). However, this base line does not adequately consider the possibility of nonimmunologic feeder or cytotoxic effects of lymphoid cells on some cells in culture. Significant cytotoxic effects by all lymphocyte preparations relative to the media control in a test against a particular cell line might indicate reactivity by all donors but also might reflect the susceptibility of the target cells to depletion of growth-promoting factors by the added cells. This susceptibility could also vary from day to day, depending on the health of the target cells. We have examined several other types of controls for possible use as standard base lines in the isotopic release assays: unlabeled autologous or other tissue culture cells and X-irradiated lymphocytes. None of these have given sufficiently consistent results. We have therefore decided to use several normal donors in each experiment and to select the results of the least active normal as the base line (Oldham RK, Herberman RB, Djeu JY, et al: Submitted for publication). This controls for nonimmunologic effects of lymphocytes and also allows for the detection of normal reactivity as well as patient reactivity in the test. The presence of lymphocytes in the well reduces the amount of day-to-day variation in control levels with a given target cell. Particularly with the iododeoxyuridine assay, there is a considerable amount of fluctuation in media control values between tests. However, even the least active normal base line is not entirely satisfactory, since it is still subject to the variation in the range of cytotoxic reactivity of the few normal donors on a given day. It will probably be best to use a standard source of nonreactive or poorly reactive normal donor cells as the base line. Cryopreserved lymphocytes from the same individual may be suitable for this purpose.

The type of target cells used in the assays is often cited as a major factor influencing the results [*see* "Discussion" in (*17*)]. Most studies of the Hellströms, showing histologic type-specific reactivity, were performed with target cells in culture for only short periods (i.e., a few days to a few weeks). It has been suggested that only these short-term cultures are suitable as target cells and that tests with established cell lines show considerably less specificity and more normal reactivity. However, in one study pointing to such differences, only 12 normal donors were tested, and 2 reacted with short-term cultures as well as established cell lines (*22*). Also, in this work and in two other studies showing no consistent difference between short-term and long-term cultures [(*12*); Fossati G, Canevari S. Della Porta G: Personal communication] the media control was used as base line. Therefore, as indicated above, some observations might be due to nonimmunologic, tissue culture effects rather than to the presence or absence of normal reactivity. When we compared the results obtained from short-term cultures with those obtained from established lines, using the least active normal donor as the base line, we found more cytotoxic reactivity by both patients and controls against the established lines, but we also noted a considerable amount of normal reactivity and a lack of histologic type-specific reactivity with the short-term cultured target cells (Oldham RK, Herberman RB, Djeu JY, et al: Submitted for publication). Therefore, it seems rather unlikely that this issue is critical for the disparate results between different laboratories. It is important, however, not to lose sight of the fact that not all tissue culture cells derived from tumors actually consist of homogenous populations of tumor cells. There is a continuing, vexing problem of successful growth of normal cells from the tumors, particularly fibroblasts. Whereas fibroblastic overgrowth can usually be identified morphologically by experienced investigators, the possibility of growth of other normal cells, e.g., normal epithelial cells of a type similar to that of the tumor, is more difficult to exclude. The recent ability to test for tumor development by human cultured cells in nude mice is helpful in this regard. Other methods for identifying tumor cells in culture are also being used with increasing frequency. These include chromosome analysis, search for characteristic markers on tumor cells (e.g., melanin, estrogen receptors, tumor-associated antigens), and release of tumor-associated products into culture medium (e.g., carcinoembryonic antigen, chorionic gonadotropin). However, even this cannot rule out the presence of a mixed cell population in culture. Virtually none of the current immunologic studies are being done with completely characterized, cloned target cells. In addition, some tumor cells in culture, be they short-term or established, may not contain or may lose tumor-associated antigens. This has been observed in various well-studied animal tumor systems.

Another technical aspect of the assays that varies considerably between laboratories is the separation of effector cells from whole blood. Some investigators prepare lymphocytes from heparinized blood by flotation on Ficoll-Hypaque gradients; some use defibrinated blood, sediment the erythrocytes with gelatin, and passage the leukocytes through nylon columns to remove adherent cells; others use other combinations or variations of these procedures. Contaminating granulocytes or monocytes can produce nonspecific cytotoxicity (*17*). Some workers have emphasized the adverse effects of Ficoll-Hypaque sedimentation, indicating that exposure of effector cells to this material may cause artifactual normal reactivity or nonspecific reactivity [e.g., (*19, 23*)]. However, as with the target cell issue this would not appear to be the explanation for the substantial differences between results from various laboratories. For example, the Hellströms switched from another procedure to Ficoll-Hypaque separation and they have not reported less specificity or increased normal reactivity since the change. Other studies, performed with cells separated by gelatin–nylon column, still showed considerable normal reactivity and a lack of histologic type specificity (Fossati G, Canevari S, Della Porta G: Personal communication; Oldham RK, Herberman RB, Djeu JY, et al: Submitted for publication). In addition, at the recent workshop that directly compared methods among different laboratories, the procedure used for lymphocyte separation, although having some effects on results, did not show consistently more normal reactivity or loss of histologic type specificity with cells separated by Ficoll-Hypaque gradients. Despite the failure to demonstrate important differences in results due to separation methods, all routine procedures yield a heterogeneous mixture of mononuclear cells, composed of varying proportions of T, B, and other cells. It is possible that application of the recently developed methods for isolating purified lymphocyte subpopulations will be useful in distinguishing between specific patient reactivity and normal reactivity. The studies reported to date that have utilized such procedures have been somewhat contradictory (*22, 24, 25*), but many discrepancies may be related to other technical aspects and study design, and also to differences in the separation methodologies. Some cytotoxic reactivity has been associated with non-T cells (*23, 26*), and it has been suggested that cytotoxicity associated with such cells is actually antibody-dependent, cell-mediated cytotoxicity. However, no clear documentation of this, e.g., by direct demonstration of the antibodies involved or inhibition by materials known to inhibit this mechanism (immune complexes, antiglobulin reagents, aggregated γ-globulin), has been provided.

Specificity of Microcytotoxicity Assays and Occurrence of Normal Reactivity

As summarized above, none of the identifiable technical features can be clearly correlated with the pattern of results obtained in the microcytotoxicity assays. We must therefore consider seriously that normal human cytotoxic reactivity is a real phenomenon and not particularly rare, and also that the reactivity of lymphocytes from cancer patients may not be entirely directed against histologic type-specific, tumor-associated antigens. The study design of experiments in different laboratories should then be carefully examined as a central factor in the controversy. Most reports of microcytotoxicity tests with human tumor systems have been summaries of varying numbers of small, individual experiments. Each experiment typically consisted of effector cells from one normal donor and two or three target cells. The types of target cells were usually derived from the same type of cancer as the patients'; normal cells, usually skin or tissue fibroblasts, from the same or different patients; and perhaps cells derived from a different type of cancer. The normal fibroblasts have been the most frequent negative control; few cancer patients had cytotoxic reactivity against these. However, such cells may be quite resistant to cytotoxicity and are not adequate specificity controls. Experiments designed along these lines, although performed many times, would probably not detect normal reactivity and also may not be likely to document clearly the specificity of the reactions. To begin seriously to analyze the specificity of the microcytotoxicity assays, it is desirable to set up experiments involving a large "checkerboard" design, with lymphocytes from several patients with cancer of different histologic types, from patients with benign diseases of the same organs, and from several normal individuals, tested against almost an equally large array of target cells derived from various types of cancer and normal cells. All target cells used in an assay, including those used as specificity controls, need to be susceptible to cytotoxicity by lymphocytes from some patients or normal controls. The use of target cells resistant to cytotoxicity would obviously bias the control results in the desired direction of histologic type specificity. To study directly the frequency and range of normal cytotoxic reactivity, it is particularly necessary to design experiments to look for such reactivity. Several normal donors, not selected because of previous reactivity or lack of reactivity in microcytotoxicity tests, must be tested concurrently, and significant differences between their activities examined. The change in the pattern of results in some laboratories [(*12, 13*); Fossati G, Canevari S, Della Porta G: Personal communication], from apparently good specificity and no normal reactivity to considerable cross-reactivity and normal reactivity, without any changes in test methodologies, can probably be ascribed to changes in study design of the kind we have just described.

Possible Relevance of Data From Experimental Tumor Systems

It may be helpful to look to the experimental animal tumor systems for possible analogies with, and insight into, the complex and difficult-to-analyze human tumor systems. Almost all human cytotoxicity testing involves allogeneic target cells; therefore, the observed immune reactivity would have to be against common rather than individual specific antigens. In the animal tumor systems, common tumor-associated antigens have usually been related to expression of fetal or virus-associated antigens. These common tumor-associated antigens usually have not been restricted to a particular histologic type of tumor, but rather the same antigen might be found in leukemias, sarcomas, and even carcinomas. The best explanation for histologic type-specific tumor antigens might be expression of normal tissue-associated antigens on tumors, but such specificities have not, to our knowledge, been detected in cell-mediated cytotoxicity experiments in animal tumor systems. It should not be too surprising, therefore, to find cytotoxic reactivity against antigens expressed on diverse types of human tumor cells. The possibility of cytotoxic reactivity against individually specific tumor antigens, characteristic of carcinogen-induced animal tumors, needs to be evaluated by tests against autologous tumor cells. However, this is logistically a difficult task, which explains why little autologous testing has been done.

Another lesson in regard to specificity of cell-mediated cytotoxic reactions may be drawn from experiments with tumors induced by murine sarcoma virus in mice. This tumor system has been popular in experimental tumor immunology, and many studies of cell-mediated immunity in this system have been done. From limited specificity testing, it has been generally assumed that the antigens detected in cytotoxicity assays were related to murine sarcoma virus or to the serologically cross-reacting Friend, Moloney, and Rauscher leukemia viruses (*26–29*). However, by more detailed analysis of the specificity by an inhibition assay, the cytotoxicity detected by a ^{51}Cr-release assay was shown not to be related to any of these antigens but rather to those associated with mouse endogenous type-C viruses (*30*). This emphasizes the need to define carefully the specificity of cytotoxic reactions, which usually necessitates extensive testing.

Cytotoxicity by lymphocytes from some normal individuals may also be analogous to natural cell-mediated cytotoxicity recently detected in murine tumor systems. Many normal mice and rats have shown specific cytotoxic reactivity against various leukemias, lymphomas, and mammary carcinomas (*31–34*). In the systems described thus far, all natural reactivity appears to be directed against virus-associated antigens. It is intriguing to postulate that some normal human cytotoxic reactivity is directed against antigens associated with ubiquitous viruses. All such natural murine reactivity was initially missed, primarily because it was not sought and because lymphocytes from normal animals were used as the base-line control. Also, the natural cytotoxic reactivity of mice and rats against leukemia cells is mediated by a unique subpopulation of non-T cells (*31, 35, 36*) and this provides an opportunity to clearly separate normal reactivity from T cell-mediated immunity of tumor-bearing individuals.

Conclusions

Microcytotoxicity assays are presently not suitable for diagnosis of human cancer or for reliable monitoring of the course of disease of individual cancer patients. The occurrence of frequent normal reactivity and the consequent difficulties in establishing a constant base line virtually preclude both of these objectives at the present time.

Because of the technical difficulties with these assays, and the findings of normal reactivity and of lack of histologic type specificity, it is often concluded that the reactivity of some individuals is nonspecific (presumably equivalent to nonimmunologic). However, we should not be too hasty in equating failure to detect desired or expected results with failure to detect meaningful, immunologic reactions. In fact, as pointed out above, from the experience with the animal models, we probably should not have expected to find histologic type-specific, common, tumor-associated antigens. Rather than conclude that results are nonspecific and therefore discard or close our minds to such findings, we should analyze carefully these reactions and try to determine the nature of the specificity. This is not an easy task, but methods are available [e.g., the inhibition assay cited above (*30*), inhibition with tissue extracts, removal of cells reacting against a particular cell type by adsorption (*37*)] to begin such analyses.

Similarly, cytotoxic reactivity by normal individuals should not be ignored but should be directly analyzed. There are some important questions to ask: Is the normal reactivity directed against the same antigens as that of cancer patients? Does the natural reactivity reflect exposure to ubiquitous tumor-associated viruses or reflect an important, natural immune surveillance mechanism against cancer? Or, rather, is the normal reactivity some form of nonimmunologic artifact which might still be eliminated by improved technology? Is the normal reactivity mediated by the same type of cells as the reactivity of cancer patients, and if not, does this provide a basis for practical separation of these activities?

In summary, although the microcytotoxicity assays are not presently providing directly practical and useful clinical information for individual patients, we should not be overly discouraged. We believe that, rather than abandoning these tests, a concerted effort should be made to develop better and more standardized techniques and to analyze carefully the complex series of observations being made. Thus it may be possible ultimately to utilize cytotoxicity assays as valuable clinical measures of cell-mediated immunity to human tumor-associated antigens.

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ADDENDUM

In the past 3½ years since the Editorial was written, considerable progress has been made in the understanding of some of the problems associated with the measurement of immunity to human tumors by microcytotoxicity assays. Most investigators have abandoned the complex and subjective visual assays and have turned to isotopic release assays, which measure lysis of target cells more selectively. More importantly, increased attention has been devoted to the important role of natural killer (NK) cells in cytotoxicity assays with cultured human tumor cells. NK cells have come to be recognized as a particular subpopulation of lymphocytes, and their characteristics have been extensively studied in many laboratories [see (*1*) for review]. This has led to considerable controversy about some of the cell surface markers on human NK cells and the possible relationship between NK- and K-cells mediating antibody-dependent cell-mediated cytotoxicity (*2*).

Several major issues were raised in the Editorial, which warranted and received subsequent attention: 1) Do cancer patients have, in addition to NK cells, specifically immune cytotoxic T-cells? 2) Can NK cells be separated from immune T-cells of cancer patients on the basis of some differences in the characteristics of the effector cells? 3) Does NK activity reflect an important natural immune surveillance mechanism against cancer? I will briefly summarize recent information about each of these points.

Investigators have made numerous attempts to discriminate between NK cell activity and the immune cytotoxic reactivity by cancer patients. One approach, developed by Cannon et al. (*3*), has been to test simultaneously the cytotoxic reactivity of lymphocytes against a cell line sensitive to NK activity and against a target cell with antigens relevant to the cancer under study. By using a cell line derived from breast cancer and the K562 myeloid cell line, scientists could identify some patients with breast cancer who had relative hyperreactivity against the breast cancer-derived line. These data suggested that these patients had NK cells and another type of effector cell. For more definitive discrimination, it would be useful to separate NK cells physically from more selective effector cells. However, this has proved to be extremely difficult. It has not been possible to use rosetting with sheep erythrocytes or other T-cell markers, since human NK cells have low affinity receptors for sheep erythrocytes (*4*) and are sensitive to lysis by anti-T-cell sera plus complement (*5*). When it was found that human NK cells had receptors for the Fc portion of IgG and NK reactivity against K562 cells could be completely removed by adsorption onto monolayers of immune complexes (*4*), it seemed likely that this would be a good procedure for separation of NK cells from cytotoxic T-cells. However, recent evidence indicated that natural cytotoxic reactivity against some target cells can be mediated by cells lacking Fc receptors (*6, 7*). Therefore at present, it is difficult to separate NK cells definitely from cytotoxic immune T-cells on the basis of different cell surface markers. However, selective adsorption of NK cells onto monolayers of NK-sensitive target cells seems feasible, and experiments with this approach are in progress.

One possible explanation for the difficulty in demonstrating human cytotoxic T-cells reactive against tumor target cells is that the relevant antigens may be restricted to autologous tumor cells or to tumor cells having compatible major histocompatibility complex (MHC) antigens. Studies on virus-infected or hapten-modified target cells of mice (*8*, *9*) have led to the conclusion that immune T-cells can only lyse histocompatible target cells. If this also applied to immunity to human tumors, then immune cytotoxic T-cells would not be expected to react with most of the available allogeneic cell lines. It is presently unclear whether this is a major obstacle in detecting specific reactivity against human tumors. Recent information has called into question the concept of complete MHC restriction for cytotoxic T-cells. Several investigators have shown that T-cells from mice immunized by syngeneic tumors or oncogenic viruses could react to some extent against allogeneic tumors (*10–12*). Also, in the few studies on in vitro generation of cytotoxicity against human tumor cells or hapten-modified cells in which scientists have examined this point, considerable reactivity against allogeneic target cells was observed (*13–15*). Furthermore, Jondal et al. (*14*) appeared to detect reactivity of cytotoxic T-cells from a Burkitt's lymphoma against allogeneic Epstein-Barr virus-infected cell lines. Despite these encouraging indications that allogeneic antihuman tumor cell reactivity by cytotoxic T-cells may be detectable, some MHC restriction may be present and studies with autologous tumor cells may be advantageous. Moreover, fresh tumor cells appear resistant to lysis by NK cells and yet may be sensitive to immune T-cells. Vose et al. (*16*) recently described autologous antitumor reactivity that appeared to be selective and distinct from NK reactivity, but no one has yet definitively shown the effector cells to be immune T-cells. Hopefully, information will soon be available on the incidence of cytotoxicity against human tumors by immune T-cells and on the distribution of the antigens which are recognized.

Much recent attention has been given to NK cells and their possible importance in immunosurveillance against tumors (*17–19*). Although immune T-cells play a central role in in vivo resistance to tumor growth, their reactivity cannot account for all aspects of immune surveillance. Animals that have few if any mature T-cells or are otherwise immunodepressed may still have some resistance to tumors. Such animals frequently have intact or even elevated levels of NK reactivity. Furthermore, numerous instances of correlation have occurred between in vivo resistance to tumor challenge and in vitro NK reactivity. Natural cell-mediated cytotoxicity may represent one of the first lines of defense against tumors and may be particularly effective in dealing with small numbers of tumor cells at early stages of their growth (*19*). This possibility is being intensively evaluated. However, sufficient information is already available to indicate that reactivity by NK cells in microcytotoxicity assays is a real biologic event and not an in vitro artifact that can be simply ignored or discounted.

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GUEST EDITORIAL

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Alpha-Fetoprotein[3]

Striking alterations in the production of AFP associated with developmental, regenerative, and carcinogenic events have stimulated an increasing interest in this protein. AFP is a serum protein similar in size, structure, and amino acid composition to serum albumin, but it is detectable only in minute amounts in the serum of normal adults. During prenatal development, however, the circulating levels of AFP are extremely high and, at least for a short time during fetal life, it is the dominant protein species in the serum. Elevated serum concentrations of this protein can be achieved in the adult by exposure to hepatotoxic agents or hepatocarcinogens and are frequently associated with the growth of hepatocellular or yolk sac tumors.

Almost all our knowledge of AFP derives from data accumulated during the last 10–15 years. In 1957, Bergstrand and Czar (*1*), using electrophoresis, identified in cord blood an alpha protein that was not detectable in adult human serum and gave it the designation of X-component. In 1963, Abelev et al. (*2*) recognized that an alpha migrating protein, normally detected only during pregnancy or in sera of newborn mice, was identical to one in mice bearing transplanted hepatocellular carcinomas; they named this protein embryonal α-globulin. Shortly thereafter, Tatarinov (*3*) reported similar observations in a human patient with hepatocellular carcinoma. A few years later, AFP was identified in the sera of rats with chemically induced primary liver tumors (4). Since the appearance of these reports, the results of a large number of experiments have been published which describe the relationship of AFP production by hepatocytes to variations in physiologic states and pathologic alterations. The number of agents used as challenges, of animal species tested, and of clinical studies done has increased exponentially. In the early 1970's, sensitive radioimmunoassays for AFP were devised (*5–8*). These permitted much more precise quantitation and thus revealed relationships not previously noted.

Despite the vast accumulation of data, many important basic questions remain unanswered. This editorial reviews the current status of AFP studies and identifies some of the unanswered questions as well as the enigmas. Some unanswered questions on the topics discussed are listed at the end of each section.

PRENATAL DEVELOPMENT AND PREGNANCY

Serum concentrations of AFP found in the rat under a variety of normal and experimental situations are illustrated in text-figure 1. Differences occur in both the amplitude and the rate of appearance and disappearance of circulating AFP after challenge or physiologic alterations. During prenatal development, AFP is produced by the fetal liver and yolk sac (*9, 10*). AFP in maternal serum is derived by transfer from the fetus (*11*). Abnormally high concentrations of AFP in the amniotic fluid or maternal serum are associated with conditions whereby leakage or decreased turnover of fetal AFP occurs and are useful diagnostically for conditions such as neural tube defects, anencephaly, esophageal atresia, intrauterine fetal death, and congenital nephrosis (*12–15*). In the rat, after delivery, the serum AFP concentration of the mother falls, but that of the newborn rat remains elevated until 4 weeks of age when the concentration abruptly falls to adult levels (*16*). The presence of AFP in neonatal sera is associated with continued proliferation of the hepatocytes after birth. Preliminary evidence indicates that the amount of AFP produced by dividing liver cells may be influenced by hormones such as glucocorticoids (*17*). A rapid drop in AFP concentration occurs when intense neonatal liver growth ceases. The association of AFP production with hepatocyte proliferation is supported by in vitro studies of fetal rat hepatocyte cultures (*18, 19*). Despite the intensity of postnatal synthesis, the level of mRNA for AFP relative to mRNA for albumin is reported to be greatly diminished by birth in the mouse (*20*). In the human fetus the serum concentration of AFP reaches a peak of 3,000 μg/ml at 15 weeks of gestation; then it falls to approximately 20 μg/ml at

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ABBREVIATIONS USED: AFP = α-fetoprotein; VLDL = very low density lipoprotein; 2-FAA = *N*-2-fluorenylacetamide.

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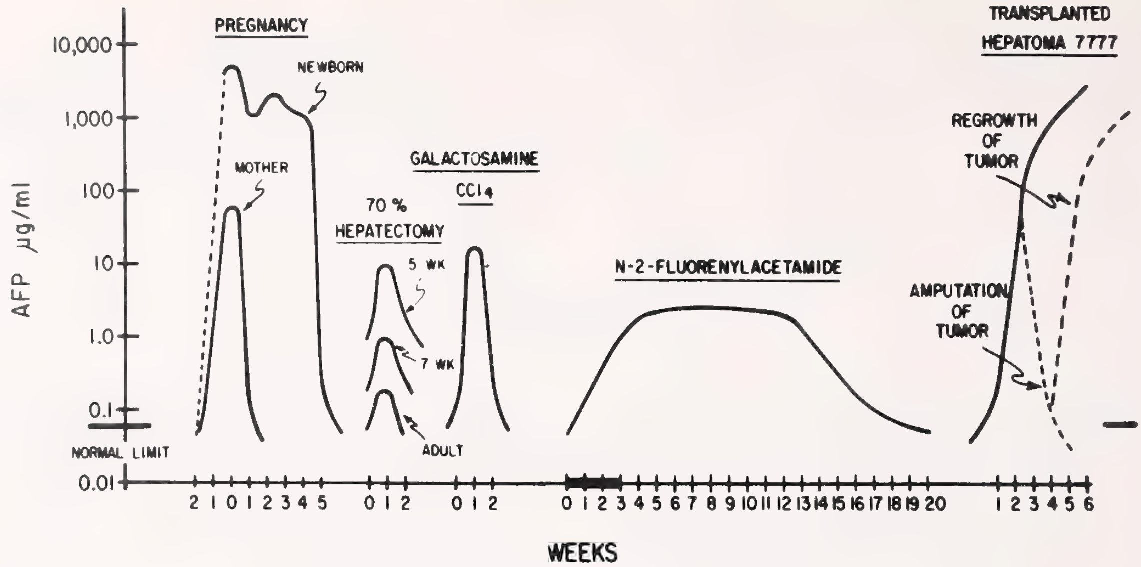
Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.



TEXT-FIGURE 1.—Serum AFP concentrations in rats.

birth and continues to fall after birth to the normal adult concentration of 0.02 μg/ml (*15, 21*). Prolonged elevations during the neonatal period suggest the hereditary diseases tyrosinemia (*22, 23*) or ataxia telangiectasia (*24*).

Despite intensive investigation, no specific function for AFP during fetal life has been identified. Four major hypotheses have been offered, each supported by some experimental data: 1) AFP functions as a fetal albumin. The concentrations of the AFP in sera of fetal and newborn animals have a reciprocal relationship with albumin concentrations (*25, 26*). Further, AFP and albumin are structurally related (*27–30*). Recent analyses have demonstrated amino acid homology between these proteins (*28*), as well as immunogenic similarities, once they are denatured (*31*). Therefore, AFP possibly has the same osmotic and carrier functions in the fetus that albumin does in the adult, and perhaps additional functions as well. 2) AFP controls the immune response. AFP has been shown to be immunosuppressive for some in vitro immune responses (*32–37*). Therefore, AFP may protect the fetus from maternal immune attack. However, an immunosuppressive effect of AFP is not supported by other studies (*38–42*), and AFP is present in the eggs of avian species, which are not susceptible to maternal immune response (*43, 44*). 3) AFP blocks estrogen effects. AFP of murine species has a strong affinity for estrogen (*45*) and might serve to protect the fetus from the effects of maternal estrogen. However, human AFP and that of other species do not bind estrogen (*46*), which raises some doubt as to the significance of this capacity. 4) Intracellular AFP is a growth-control factor. AFP may control the growth of hepatocytes via estrogen binding and inhibition of VLDL synthesis. Estradiol stimulates VLDL, and VLDL inhibits liver cell growth in vitro. Therefore, during active proliferation of liver cells, intracellular AFP may block the estradiol-stimulated synthesis of VLDL and thus permit hepatocellular growth (*19, 47*). Direct evidence supporting this hypothesis has not been obtained, and the failure of AFP of some species to bind estrogen also limits the broad applicability of this possibility. Therefore, a specific function for AFP during fetal development has not been clearly identified. Preliminary evidence of a vital function of AFP during fetal development was the finding that injection of antisera to AFP into pregnant rabbits (*48*), rats (*49*), chickens (*50*), and mice (*51*) induced abortions and fetal abnormalities. However, this result has not been reproduced in other laboratories [(*52*); Sell S: Unpublished data].

Questions

1. What is the function(s) of AFP? Is it possible that this protein fulfills a broader spectrum of activities than any single protein species in the adult, thus accounting for conflicting interpretations of its function?
2. What is the alteration, factor, or event that causes AFP synthesis to decline in the perinatal period? Is this simply a function of the number of dividing hepatocytes? What role do hormones have in regulation of AFP production?
3. Is the cessation of AFP production linked to that of other fetal proteins that diminish during this period? Is this linkage genetic in origin or causally related to other developmental changes?
4. What is the significance of the elevated AFP associated with genetically determined disorders such as tyrosinemia, a disease associated with a high incidence and an early appearance of hepatocellular carcinoma?

RESTITUTIVE PROLIFERATION

AFP production is associated with hepatocyte proliferation in the adult (*16, 25*), induced by partial hepatectomy (*53*), chemically induced liver necrosis (*54, 55*), and viral diseases such as hepatitis (*56*). As illustrated in text-figure 1, the elevation of serum AFP produced after restitutive proliferation is transient. When rats undergo 70% hepatectomy, as many as 90% of the hepatocytes may participate in cell division within 48 hours. The degree of elevation of AFP in rats after partial hepatectomy is related to age; younger rats have a higher elevation than older rats (*16*). This finding is of interest, since elevations of serum AFP were not detectable in human adults who underwent partial hepatectomy for benign disease (*57*), but a transient elevation was found in a 13-year-old boy who had a left partial hepatectomy for a hepatocellular carcinoma that did not produce AFP (*58*). These differences in "age-related elevation" may result from the number of residual hepatocytes that enter mitosis per unit time and the fluid volume of the host. After chemically induced liver injury in rats, the amount of serum AFP is directly related to the degree of liver cell injury, as determined by release of liver cell enzymes into the serum (*55*). However, the elevation found after chemically induced necrosis in adult rats is 2 logs more than that observed after partial hepatectomy (*54, 58, 59*) (*see* text-fig. 1). This implies either that chemicals induce AFP production or that the restitutive proliferation after chemically induced injury is more intense, perhaps because of a more prolonged hepatotoxic effect. Also, chemical injury might induce a reduction in AFP clearance. After injury with CCl_4, Engelhardt et al. (*60*) found AFP-containing cells prior to the onset of liver cell proliferation, i.e., during the first cell cycle. However, the major serum AFP elevations coincide with liver cell proliferation and are a good indication of both the degree of injury and the prognosis (*55, 56, 61*). Absence of AFP elevation after acute liver injury indicates necrosis so extensive that insufficient liver cells have survived from which restitutive proliferation can be originated. Thus recovery is unlikely.

Phenobarbital injection in rats also results in a significant elevation of serum AFP within 6 hours (*62, 63*). This early elevation, which occurs prior to hepatocyte proliferation, suggests that AFP release (production) may occur as the result of hypertrophy without cell division. This elevation is very slight and transient and is followed by an even smaller elevation at 48 hours, probably secondary to proliferation.

Questions

1. Is the production of AFP that is associated with hepatocyte division a result of gene derepression and transcription or an alteration in mRNA transport and/or translation?
2. What accounts for the relatively low level of AFP in the stimulated adult when compared with fetal liver production?
3. Why does exposure to hepatotoxic agents result in markedly higher elevations of serum AFP than does partial hepatectomy?

HEPATOCARCINOGENESIS

A number of investigators have reported that exposure to chemical hepatocarcinogens evokes a responsive synthesis of AFP (*59, 61, 64–73*). With new experimental findings, the relationship between AFP production and hepatocyte alteration during chemical carcinogenesis has become even more complicated. The prolonged elevations of AFP after subcarcinogenic doses of some hepatocarcinogens probably indicate a major difference between the effects of these agents and hepatotoxins. This cannot be determined precisely, since the effects of more extended exposure to the group of toxic agents have not been reported. However, in an ongoing study prolonged elevation of AFP has not been found after 2 months' repeated exposure of rats to CCl_4 or galactosamine (Sell S: Unpublished observations). Significant elevations of serum AFP are produced in female rats of strains reported to be "metabolically resistant" to the carcinogenic effects of 2-FAA (*71*). In addition, the elevation of AFP produced in male rats by doses of 2-FAA estimated to be in the range of 1/1,000 of a carcinogenic dose occurred with neither morphologic nor chemical evidence of cell damage (*71*). Noncarcinogenic analogs of several hepatocarcinogens, themselves nontoxic to hepatocytes, failed to induce any elevations in AFP regardless of the duration of administration or, within reasonable levels, the dose. These results emphasize the difficulty in distinguishing the mechanism of increased serum AFP concentrations, either by experimental data or, conceptually, between exposure to hepatotoxins and exposure to carcinogenic agents.

A number of investigators who have used semiquantitative techniques have reported that the administration of hepatocarcinogens produces elevation of serum AFP concentrations during the evolution to malignancy (*64–67, 69*). Quantitative radioimmunoassays show that serum concentrations of circulatory AFP rise shortly after hepatocarcinogen ingestion and usually level off despite continued administration (*71, 72*). This elevation frequently occurs prior to any significant alteration in morphology and then subsides despite continued administration. A subsequent elevation of AFP may occur, in the absence of detectable malignancy, if the administration of carcinogen is continued beyond a carcinogenic dose (*71, 72*); these elevations also subside. However, because secondary elevations do not occur if administration of the carcinogen is halted when a carcinogenic dose has been obtained, their significance is uncertain. The final elevation of AFP is usually associated with the appearance of malignant cells and usually progresses with tumor growth and metastasis.

Although it has been suggested that the initial elevation of AFP which results from exposure to carcinogens represents one phase of a specific sequence of gene alteration related to carcinogenic evolution, and data are available to support this contention (*71, 72*), strongly conflicting data are also available. The decline of the initial elevation of AFP occurs during administration of a carcinogenic dose of 2-FAA and well before the appearance of the putative premalignant lesions, the so-called neoplastic nodules (*71*). An elevation of AFP similar in amplitude and in duration occurs with administration of a subcarcinogenic dose of the same carcinogen. Further, double diffusion in agar has previously indicated (*68*), and use of radioimmunoassay in our laboratories has recently confirmed, that no elevation of AFP occurs during the initial weeks of exposure to the powerful hepatocarcinogen diethylnitrosamine. Elevations are detected only when approximately the total carcinogenic dose has been ingested. From this point in exposure, the elevation of AFP is progressive and appears to coincide with the appearance of foci of malignant cells.

Although these findings have clouded the significance of the initial response of AFP to hepatocarcinogens, they have reinforced the concept that the final elevation is the direct result of synthesis by malignant cells. This relationship is supported by the finding that elevations of the serum concentration of AFP in mice with a high predilection to spontaneous hepatocellular carcinomas do not occur with aging, but only in association with detectable tumors (*74*). Thus, despite the obviously increasing risk of malignancy and a reasonable assumption of progressive cellular evolution toward malignancy with aging, AFP production increases only when hepatomas develop. In addition, the BALB/cJ mouse maintains serum AFP concentrations tenfold higher than those maintained by other strains, without any detected increase in the incidence of hepatoma (*75*, *76*).

The nature of the cell type(s) producing AFP after exposure to hepatocarcinogens is unclear. For instance, periportal "oval" cells appear after administration of certain hepatocarcinogens such as 3′-methyl-4-dimethylaminoazobenzene. These oval cells contain AFP when reacted with specific immunofluorescent reagents (*70*). A similar observation has been made in choline-deficient rats fed ethionine, which have tremendous oval-cell hyperplasia (Shinozuka H, Lombardi B, Sell S, et al: Unpublished observations). However, elevated serum concentrations of AFP also occur after 2-FAA administration when few, if any, "oval" cells can be identified in the liver (*71*). Some evidence indicates that AFP may be identified in cells of some hepatic nodules that are found prior to the appearance of frank hepatocellular carcinomas (*77*), but it has still not been determined if such nodular cells or oval cells are the direct precursors of the carcinomas that develop.

Questions

1. What is the significance of the initial elevation of AFP that results from exposure to many carcinogens? What is its relationship to that induced by hepatotoxins?
2. Is the synthesis of AFP part of an integrated process of gene derepression induced by carcinogen exposure?
3. What accounts for the absence of elevated levels of AFP in rats during their early exposure to diethylnitrosamine? Can some carcinogens act as derepressors whereas others cannot? Does this failure suggest that stimulation of AFP synthesis is a parallel process not necessarily related to carcinogenesis?
4. What cell type(s) is stimulated to synthesize AFP by carcinogen exposure? Is it a specific subpopulation?
5. Do humans have elevated serum AFP concentrations after hepatocarcinogen exposure, prior to development of hepatoma?

HEPATOCELLULAR CARCINOMA

Production of AFP by hepatocellular carcinomas is consistent with the numerous theories that attribute the carcinogenic process to regression or incorporate reexpression of fetal characteristics as an integral feature. Synthesis of these substances is exemplified by the reappearance of fetal isoenzymes and other proteins such as carcinoembryonic antigen (*78*). Recapitulation of developmental surface antigens (*79*) and of the susceptibility of these cells to lectin agglutination has also been demonstrated (*80–82*). However, the production of different developmental products by tumors does not appear to be coordinated. Thus tumors that produce carcinoembryonic antigens do not usually produce AFP, and fetal liver isoenzyme patterns are not clearly associated with the ability of a hepatoma to produce AFP (*78*). Unfortunately, no available published results attempt to correlate these various modalities directly in adequate detail. In one study (Becker FF: In preparation) no correlation was apparent between AFP production and susceptibility of hepatoma cells to lectin agglutination, nor was the former found to be related to the metastatic potential of the tumor.

Different "levels" of expression of AFP by various types of tumors must be considered (*78*). The ability of the tumor to synthesize AFP usually reflects that of the fetal tissue from which its normal progenitor arises (first level). In the fetus, the liver and yolk sac are the primary organ sites of AFP synthesis; minimal amounts are produced by the fetal gastrointestinal tract (*9*, *10*, *21*, *83*). AFP production in adults is associated with up to 80% of primary hepatocellular carcinomas and with essentially all teratocarcinomas that contain yolk sac analog elements (endodermal sinus tumors) (*13*, *24*, *25*, *84–92*). A much less frequent association is found with pancreatic and gastric tumors (second level), and an even lower frequency with pulmonary adenocarcinomas (third level) (*85*, *91*). These tumors are derived from gastrointestinal and bronchial endodermal embryonic tissues, respectively, and are, therefore, embryologically related to the yolk sac endoderm. Finally, elevated serum concentrations of AFP have very rarely been associated with nonendodermal tumors such as leukemia and myeloma (*93*). If verified this would mean production at a fourth level. However, most investigators have found AFP to be associated only with tumors derived from yolk sac endoderm (hepatomas or teratomas). Production of AFP by human tumors arising from related endoderm, such as gastrointestinal or pulmonary tumors, has not been critically documented, since other reasons for AFP elevation were not ruled out in the reported studies and direct studies of synthesis by nonhepatoma or non-yolk sac tumor tissue have not been reported. In addition, chemically induced pancreatic or gastrointestinal tumors in animals have not been associated with elevated serum AFP concentrations (Longnecker D, Weisburger E, Sell S: Unpublished observations).

Although AFP synthesis has been related by some workers to tumor growth rate or to histologic "differentiation" of hepatomas (*94*), experimental evidence in the rat suggests that the chromosome composition of the primary tumor cells is more important. Thus aneuploid tumors are frequently associated with AFP production, whereas diploid tumors are not (*95*). This association persists for many generations despite significant alteration in karyotype from one generation to another (*96*). The metastatic foci of these tumors reflect the production rate of the primary tumor. Only in selected instances, such as the Morris tumor 7777 with a single marker chromosome, is the relationship to significant aneuploidy clearly broken, in that this near-diploid tumor produces extraordinary quantities of AFP (*97*).

Some evidence suggests that the consistency of AFP production by hepatocellular carcinomas may depend on the chemical agents used to induce them. Thus tumors resulting from exposure to 2-FAA have an extraordinary variation in AFP production ranging from none to near fetal levels, whereas those induced by diethylnitrosamine are almost all strongly productive, even though subcarcinogenic doses do not produce serum AFP elevations. This leaves many unanswered questions concerning AFP production and tumor characteristics.

Questions

1. Are those cells which produce AFP after exposure to carcinogens precursors of the cells that eventually give rise to the hepatoma, or do they represent different populations?
2. Why is production of AFP by the resultant tumors so diverse despite the invariable stimulation of AFP production that occurs after administration of a single carcinogen to rats?
3. What percentage of nonproductive tumors, as determined by circulating levels of AFP, are productive but nonsecretory?
4. Is the variable amount of AFP produced by these tumors a result of differences in the percentages of productive cells or the level produced by each cell? If the former pertains, what is the biologic nature of the tumor-cell subpopulations, i.e., are they both malignant? If the latter pertains, how is this mechanism modulated in an "autonomous" cell?
5. Can tumors derived from nonendodermal origin produce AFP? If so, what is the mechanism controlling this level of gene expression?

AFP IN HUMAN DIAGNOSIS AND PROGNOSIS

Application of experimental findings on AFP to the study of human disease has been rapidly expanding. Serum AFP concentrations have been used to follow tumor growth and the effects of therapy in experimental animals (*98–100*) and in humans (*85, 101–106*). The normal serum concentration of AFP in humans is 20 ng/ml. High serum concentrations of AFP in an adult (>1,000 ng/ml) are almost always the result of production by tumor. However, since elevations of serum AFP may also be found in patients with viral hepatitis or cirrhosis whereas some patients with hepatomas or teratocarcinomas do not exhibit elevated serum AFP, a low elevation is not a reliable diagnostic marker. To evaluate the clinical significance of serum AFP, serial determinations must be performed. Low, transitory elevations are usually found with liver injury; higher, sustained, or increasing elevations are more typical of production by tumors. As mentioned, normal AFP concentrations with evidence of acute liver injury indicate a poor prognosis (*107*), because elevated serum AFP concentrations probably reflect restitutive proliferation.

Prolonged elevation of serum AFP concentrations in humans has been associated diagnostically only with tumor burden. The possible significance of elevated serum AFP concentrations in populations with presumptive exposure to hepatocarcinogens has not been fully explored. Purves et al. (*102*) reported that the serum AFP concentrations of the Bantu and of males from Mozambique and Malaysia, who have a higher incidence of primary hepatocellular carcinoma, were higher than the concentrations in Caucasians. However, the significance of this observation in regard to racial differences, hepatocarcinogen exposure, or endemic hepatitis remains unclear. The data indicate a fall in the serum concentrations of the Bantu after 1 year of habitation in South Africa, during which time they were exposed to a diet that differed from their native one. This suggests that an environmental rather than a racial difference was responsible for AFP elevations. After a systematic survey of males from seven populations at apparently different risk for hepatoma development, no conclusive demonstration of a relationship between serum AFP concentrations and hepatoma incidence among the groups was found (*108*). Women who use hormonal contraceptives do not have elevated serum AFP concentrations and in general develop benign lesions, not hepatocellular carcinomas (*109*). Clearly, more quantitative data from human studies are required to determine if serum AFP concentrations may be useful in detecting hepatocarcinogen exposure. In an extensive study in the Republic of China, 75 cases of asymptomatic primary carcinoma of the liver were detected in a screening test based on serum AFP concentrations of more than 490,000 persons; only 7 false positives were recorded.[4] Thus no general elevation of AFP occurs in populations at high risk, and it appears that AFP production is associated with early stages of liver cancer in humans.

The illustration of so many major and perplexing questions related to the study of AFP should be as stimulating as it is humbling. We think experiments that are aimed at answering such questions will have significance in many areas of oncology, as well as implications far beyond the study of malignancy. These would include fetal development, liver injury, reparative cell division, and perhaps control of functional aberration in, and control of, the proliferation of normal and malignant cells. We have reached that point in the study of this protein when we must focus our attention on major issues rather than continue to collect confirmatory but not necessarily contributory data. We hope that the questions posed in this editorial will help focus future experimental and clinical studies on AFP.

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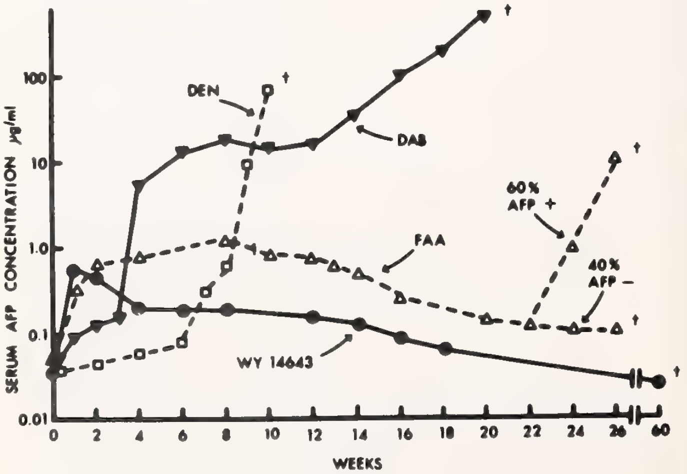
ADDENDUM

Results obtained since the original publication of this Editorial show that different carcinogens given to rats produce different 1) kinetics of serum AFP elevations, 2) cellular alterations in the liver, and 3) populations of AFP-containing cells. Thus it is possible that different chemical carcinogens may induce hepatocellular carcinomas that evolve by different cellular pathways. Previously a sequential alteration of liver cells, from foci of altered hepatocytes through reversible neoplastic nodules to frank hepatocellular carcinomas, was postulated as a common pathway activated by various carcinogens. However, four patterns now seem apparent (text-fig. 1). Diethylnitrosamine given orally at low but carcinogenic doses produces no early AFP elevations nor morphologic change. After a dose-dependent time of exposure, there is a rapid progressive increase in serum AFP concentrations associated with the appearance of multiple hepatocellular carcinomas in all treated animals. Diaminoazobenzene produces a three-stage elevation of AFP. In the first week, necrosis of the liver and a slight AFP elevation associated with restitutive proliferation are observed, followed after a few weeks by a more prolonged elevation associated with oval cell and nodule development. After AFP levels reach a plateau for another 6–8 weeks, they again rise steadily but are now associated with the appearance of hepatocellular carcinomas. *N*-2-acetylaminofluorene and ethionine produce an immediate moderate elevation of AFP associated with little or no morphologic change. Even with continued administration of the carcinogen, the serum AFP concentrations fall to just above normal levels. The serum concentrations begin to decline at the time of maximum neoplastic nodule formation. After 22–36 weeks, hepatocellular carcinomas develop which may (~ 60%) or may not (~ 40%) produce serum AFP elevations. WY-14643 and azaserine produce early hepatocyte proliferation without necrosis but associated with a moderate temporary AFP elevation. Later, the serum AFP concentrations return to normal even though neoplastic nodules appear.



Text-figure 1.—Serum AFP concentrations in rats exposed to different carcinogens.

Although the numbers of animals studied so far are limited, all the tumors have been AFP nonproducers. Three new cell populations may be identified as containing AFP after exposure of rats to hepatocarcinogens: oval cells, bile duct-like cells, and small zones of "atypical hyperplasia." Neoplastic nodules do not contain cells with demonstrable AFP. The foci of altered hepatocytes which contain gamma glutamyl transpeptidase do not contain cells which label for AFP. Therefore, the conclusion can be drawn that hepatomas induced by chemicals in rats may develop by different cellular pathways including foci of altered hepatocytes, oval cells, cells in atypical hyperplastic zones, cells in neoplastic nodules, or even from normal-appearing hepatocytes. Since neoplastic nodules are AFP− and the other new cell types AFP+, one may tentatively conclude that AFP− carcinomas arise from nodules, whereas AFP+ tumors arise from other cell types. However, no direct evidence supports this hypothesis. At the present time, it is not possible to determine if one or more of these cell types is the precursor of the malignant tumor.

These studies were performed in collaboration with Hisashi Shinozuka, Benito Lombardi, and Janarden Reddy.

GUEST EDITORAL

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"While writing this editorial, I was shocked and saddened to learn of the untimely death of Dr. John P. Glynn with whom I worked for two years. Cancer research, advanced by his life, has lost greatly by his death. I therefore dedicate my editorial to the memory of Dr. John P. Glynn."

Immunotherapy of Cancer

TUMOR IMMUNOTHERAPY is progressively becoming an important and even fashionable area of investigation due to an ever-increasing body of evidence implicating immunologic factors in human as well as animal oncogenesis. The critical bit of evidence—and the *sine qua non* for any consideration of clinical immunotherapy—has been the demonstration that human tumors, like animal tumors, possess tumor-specific antigens (TSA) not present (qualitatively or quantitatively) on nontumor tissue.

In animals, the tumor antigens are detectable by their ability to elicit an immune response that can be measured *in vitro* and corroborated *in vivo*. By contrast, human TSA can be detected only if the patient is already reacting against his own tumor and only by *in vitro* assays which are *assumed*, but not known, to reflect an immunologic reaction operative *in vivo*. Consequently, recent advances in this area can be attributed to more extensive use of well-known *in vitro* assays for humoral antibody against human tumors and to the development of tests for cellular reactivity against them. Unfortunately, the relationship between the tumor antigens or the responses detected against them by one test and those detected by another remains largely unclear because each investigator tends to "do his own thing" with one test and with the human tumor of interest to him.

Nevertheless, *in toto*, the evidence for the existence of human TSA is impressive and raises the possibility of immunotherapy directed against such antigens as a way to preferentially or selectively kill residual tumor cells with a higher degree of antitumor specificity than can be achieved with even the best chemotherapy. The magnitude of the task, however, is perhaps most cogently suggested by the fact that, despite many years of knowledge of animal TSA and the reactions which they evoke, immunotherapy of established tumors is rarely successful, even in easily manipulated animals. Although every immunologic approach has been effective in some animal tumor systems if instituted *before* tumors have become clinically detectable,

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Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors will welcome suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

with rare exceptions, no immunologic approach has been effective if instituted *after* tumors have become clinically detectable. Some encouragement can be derived, however, from recent reports that some animal tumors can be eradicated by the use of immunotherapy as an adjunct to other modes of therapy.

Accordingly, I would like to briefly review and comment on several approaches to immunotherapy of established tumors in animals and man. Only the problem of eradicating cells already transformed to the malignant state will be discussed; the problem of prophylaxis, or interference with the process of malignant transformation, or eradication of the oncogenic agent—which may be the limiting factor in tumor immunotherapy—will not be discussed.

The immunotherapy approach receiving the most attention has been active immunotherapy with bacillus Calmette-Guérin (BCG) or other nonspecific stimulants of the reticuloendothelial system known to enhance immunologic reactivity against various antigens. Such stimulants can interfere with the growth of animal tumors if administered before, but not after, a tumor is clinically detectable. Comparable results have been obtained in animals treated with vaccines prepared from antigenic tumor cells. Both approaches, *i.e.*, nonspecific or specific active immunotherapy, are thus capable of inhibiting a small number of tumor cells—a number which should vary from tumor to tumor, partly depending on the response which the tumor evokes against itself and on its sensitivity to immunologic attack. Both approaches, however, for reasons still unknown, have often induced enhancement rather than inhibition of tumor growth in animals—especially of nonlymphoid tumors.

More impressive results have recently been observed in rodents with active immunotherapy used in conjunction with chemotherapy or surgery, but the precise determinants of success—other than tumor load—have not been extensively explored. The critical need now is not for additional confirmatory reports, but for more fundamental studies aimed at dissecting the *in vivo* phenomena observed by correlative *in vitro* studies of the various components of the immune response and how they are influenced by tumor growth, by conventional therapy, and by immunotherapeutic manipulations.

By analogy with the animal studies, Mathé attempted active immunotherapy in patients with acute lymphoblastic leukemia in remission after chemotherapy. Twenty patients treated with killed leukemic cells, with BCG, or with both remained in remission longer than did 10 untreated patients. Attempts to confirm these encouraging results with more patients are certainly warranted. To date, an English study, with a somewhat different BCG somewhat differently administered, has failed to confirm Mathé's results.

Many other investigators during the past 50 years have "vaccinated" patients with preparations of their own tumor—irradiated, homogenized, conjugated with foreign proteins, etc.—without convincing clinical benefit and without evidence of enhanced tumor growth. Since one of the potential advantages of a tumor vaccine over BCG is the assumed specificity of the immunogen (*i.e.*, that the tumor vaccine used contains TSA), well-controlled immunotherapy studies in man should ideally include (but to date have not) a control group of patients immunized with a comparable vaccine from comparable *nontumor* tissue. Indeed, such controls are also indicated in all studies aimed at *specifically* increasing antibody production against autologous tumors by immunization with tumor vaccines. Documentation, *e.g.*, by absorption of specific antitumor antibody, of the antigenicity of any tumor vaccine used, would also be desirable.

From the above considerations, a prototypic active immunotherapy study aimed at eradication of a small number of residual tumor cells might involve immunizing patients undergoing limb amputation for osteogenic sarcoma with their own lethally X-irradiated (demonstrably antigenic) tumor cells, with their irradiated normal cells, with BCG, or with nothing and following the time to detectable pulmonary metastases. The cellular and humoral reactivity of each patient to his tumor and to unrelated antigens should be serially tested as a function of his clinical course and of his conventional and experimental therapy.

Similar specific or nonspecific active immunotherapy studies would be appropriate and feasible in other types of patients whose tumor load has been significantly reduced by nonimmunologic modes of therapy but who have a high probability of recurrence or progression within a relatively short time. For example, patients undergoing mastectomy for breast carcinoma with four or more microscopically involved nodes or patients undergoing resection for anaplastic bronchogenic carcinoma should be considered. Similarly, patients whose solid tumor burden has been significantly reduced by any form of therapy but in whom the response is expected to be transient—*e.g.*, patients with testicular or ovarian carcinoma or disseminated neuroblastoma—should also be considered for study if some of their tumor and nontumor tissue has been stored for vaccine preparation. Finally, groups of patients with hematologic malignancies in chemotherapy-induced remission of relatively predictable duration, as observed with Hodgkin's disease, acute myeloblastic leukemia, and Burkitt's lymphoma, are perhaps most suitable for attempts at active immunotherapy and indeed for virtually all other approaches to tumor immunotherapy.

Another approach to immunotherapy, *i.e.*, serotherapy, has been attempted in patients with various disseminated tumors, usually without adequate controls and with generally unimpressive clinical results. Although there are enough reports of significant effects of serotherapy against some animal tumors to justify well-controlled clinical studies with sera from patients in remission, our enthusiasm is tempered (perhaps too greatly) by reports of enhanced tumor growth in animals after serotherapy and by the recent demonstration by the Hellströms that factors (*i.e.*, blocking antibody) present in the serum of tumor-bearing animals and man can interfere with a cellular reaction against tumor cells *in vitro*. Nevertheless, since enhanced tumor growth in animals and blocking antibody in animals and man have been reported largely or solely with *nonhematologic* tumors, well-controlled, double-blind serotherapy trials are warranted in patients with leukemia or lymphoma in remission. The trials might involve infusion of massive amounts of normal or remission serum with or without concurrent chemotherapy.

Since rejection of antigenic tumors is largely mediated by a cellular immune response, many attempts have, of course, been made to influence the growth of animal tumors by adoptive immunotherapy with identical (syngeneic) or foreign (allogeneic) lymphocytes. Syngeneic lymphocytes can usually eradicate a small number of tumor cells, provided the lymphocyte donors have been previously immunized to TSA, whereas, with rare exceptions, cells from foreign donors have most often been ineffective, probably because they are rejected too rapidly by the host before they can exert an antitumor effect.

The apparent prerequisites for successful adoptive immunotherapy in animals, *i.e.*, viable immune lymphocytes which persists at least transiently in a host bearing a small tumor load, may explain the lack of effect observed with lymphocytes infused from *normal* identical or foreign donors into patients with *widespread* tumors. The most extensive studies aimed at using immune cells have involved cross-immunization with killed tumor cells (never killed normal cells) between patients with the same type of tumor followed by cross-transfusion of white blood cells. Although the lymphocytes infused are probably immune to TSA, the lymphocytes are likely to be rejected rapidly by the host because of the sensitization against donor histocompatibility antigens.

Some of the prerequisites outlined can be met by the use of adoptive immunotherapy as an adjunct to other modes of therapy, as most vigorously exemplified by bone marrow transplantation. Unfortunately, animals and patients with leukemia treated with lethal whole-body X irradiation plus bone marrow from normal identical twins have experienced disappointingly short remissions, which suggests that nonimmune identical bone marrow is immunotherapeutically ineffective against residual tumor cells. On the other hand, foreign bone marrow in X-irradiated animals can destroy residual leukemic cells, but usually kills the animal via a graft-versus-host (GVH) reaction which cannot be controlled once it is established, but which is unlikely to exert an antitumor effect if it is effectively controlled before it is established. Attempts at bone marrow transplantation into HL-A-compatible patients with acute leukemia in relapse have shown that engraftment (after lethal X irradiation or administration of cyclophosphamide) is not a major problem, that the GVH reaction, although modified by very early immunosuppressive therapy, remains a significant and occasionally fatal problem, and that, although a remission is the rule, leukemia usually recurs rapidly, which suggests an immunotherapeutic failure. The probability of successful immunotherapy with this approach would undoubtedly increase if the immunotherapy were initiated in patients who have a small tumor load and who are clinically better able to tolerate the procedures, *e.g.*, patients with acute myeloblastic leukemia in remission. Concurrently, studies in animals should be directed at finding (*in vivo* or *in vitro*) ways to confer preferential antitumor specificity onto the GVH reaction, so as to either increase the reactivity of the foreign lymphocytes against tumor cells or decrease their reactivity against normal host cells.

A less vigorous approach to adoptive immunotherapy of established tumors, as studied in mice, has involved treatment of tumor-bearing mice with nonlethal, noncurative cyclophosphamide followed by infusion of syngeneic, hemisyngeneic, or H-2-compatible lymphocytes. The drug serves both to decrease the tumor load and to suppress host reactivity, which thereby delays rejection of infused foreign cells. This approach, *i.e.*, "adoptive chemoimmunotherapy," has been effective against two disseminated murine lymphomas and a sarcoma, but only if the infused lymphocytes were viable, were at least transiently persistent in the host, and were derived from donors preimmunized against tumor-associated antigens.

The formulation of an analogous chemoimmunotherapy approach to the treatment of tumors in man presents as one of its principal obstacles the necessity for lymphocytes already immune to TSA. Donors must, therefore, be either already reactive, as reported for relatives of patients with neuroblastoma and osteogenic sarcoma, or must be immunized *de novo* with a preparation which is likely to sensitize not only to TSA but also to normal host isoantigens and thus increase the probability of significant GVH disease.

Although a solution may be derived from future studies of immunization to TSA *in vitro*, one conceivable way to overcome the obstacle is suggested by studies indicating that the lymphocytes of almost every patient with almost any tumor are reactive against the patient's own tumor *in vitro*. Consequently, the requirement for immune lymphocytes for chemoimmunotherapy may be met clinically by using the *host* as his own *donor* of immune lymphocytes. This is reasonable only if the lymphocytes are not exposed to the immunosuppressive effects of the drug used. Accordingly, one testable approach to chemoimmunotherapy in man may be to remove temporarily some of the autologous "immune" cells, administer a high but nonlethal dose of a drug, reinfuse the autologous lymphocytes (not exposed to the drug) after the active drug has disappeared, and compare the resultant therapeutic benefit with that observed in comparable patients treated with the drug but not with the lymphocytes.

Autologous lymphocytes from various sources may be used, since no comparative data are as yet available concerning the immunologic reactivity of human lymphocytes from blood, thoracic duct, etc. If relatively fresh, nonfrozen lymphocytes are deemed necessary, then a drug with a short biologic half-life, such as cyclophosphamide, would be essential. However, if frozen-and-stored lymphocytes can be shown to retain immunologic reactivity and, therefore, can be used, multiple lymphocyte collections would be possible and the lymphocytes could be reinfused at *any* time and after *any* treatment. This would make it possible to store lymphocytes from patients before any nonimmunologic therapy is initiated, to apply chemoimmunotherapy only to those patients who have responded to such nonimmunologic therapy, and to compare the fate of responders who receive the additional high-dose chemotherapy plus the stored lymphocytes with that of responders who receive only the high-dose chemotherapy without cells or the cells without the chemotherapy.

Most appropriate for the study are patients with a predictably poor prognosis on conventional therapy, with a small antigenic tumor load against which autologous lymphocytes are demonstrably reactive and which is sensitive to the drug used. All types of patients mentioned above as being appropriate for active immunotherapy are also suitable for chemoimmunotherapy. A patient with acute myeloblastic leukemia in his first remission on conventional chemotherapy is perhaps *most* appropriate for the chemoimmunotherapy approach because his tumor load in remission is relatively small, the remission is liable to be of very short duration, his lymphocytes in remission are reportedly reactive immunologically against his tumor cells, and his tumor is liable to be somewhat sensitive to a high dose of cyclophosphamide. Thus, adults with acute myeloblastic leukemia in remission can be treated with a high dose of cyclophosphamide plus their own lymphocytes or with the drug alone, and the duration of remission in the two groups of patients can be compared. As in the proposed studies of active immunotherapy, attempts at chemoimmunotherapy should be accompanied by correlative *in vitro* immunologic tests that would give sufficient information concerning immunologic reactivity, suppression, and reconstitution to provide guidelines to subsequent protocols. One virtue of the chemoimmunotherapy approach, as opposed to other approaches, is that it assures patients of high-dose and possibly effective chemotherapy, so that they are not totally dependent on the validity of our theories or on the hoped for effect of immunotherapy.

Totally different approaches to immunotherapy may have to be considered if and when blocking antibody is shown in animals to be operative *in vivo*, *i.e.*, that it can interfere with the immunotherapeutic efficacy of immune cells *in vivo*, and especially when double-blind studies confirm and extend the impressive association which the Hellströms have observed between tumor load and blocking antibody in animals and man. The approaches may take the form of somehow removing blocking antibody, enhancing the production of competing antibody, or conceivably using chemotherapeutic or other agents when such agents are shown in experimental systems to have an adverse effect on blocking antibody formation or activity.

Although as both clinicians and researchers, we would ideally prefer to obtain much more information before attempting immunotherapy of cancer in man, in our role as clinicians we are continuously confronted with a sense of urgency to try *something* by the daily reminders that, although conventional forms of therapy are effective in some patients, they are unfortunately ineffective in many others. Accordingly, while animal tumor immunotherapy models are being developed and studied—not necessarily to develop approaches directly transferrable to a clinical situation but rather to establish *principles* of immunotherapy—some approaches to tumor immunotherapy can and should be explored in man.

Although the approaches will be dictated largely by what is already known, they would be strongly influenced by results obtained from additional prospective clinical studies to assess various immunologic responses of patients to their tumors and to unrelated antigens as a function of the clinical course of their disease and their response to conventional therapy. Ideally, such studies should involve exchange of materials, such as tumor cells, lymphocytes, serum, etc., among different laboratories so that immunologic reactivity may be tested double-blind by a variety of assays. Such exchanges should be strongly encouraged and supported so that the relationship between the immune responses measured by various tests could be determined. It might be very important to know, for example, the relationship between antimelanoma or antisarcoma antibody as detected by immunofluorescence or complement fixation by Morton and by Lewis and that detected by the cytotoxic or blocking assay of the Hellströms. Also is the antibody against carcinoembryonic antigen detectable by Gold active *in vitro* as a cytotoxic or blocking antibody? Finally, it might be crucial to determine how antibodies detected by *all* of these assays relate to the clinical course of the malignancy and to the response to therapy. Such studies might be facilitated by technical workshops to teach the various assays to interested investigators.

In the meantime, however, some approaches to tumor immunotherapy can and should be explored in patients such as those mentioned above, but only if the studies are rigidly designed and well controlled and are accompained by correlative *in vitro* tests. Unfortunately the patients who are most likely to respond to immunotherapy and most suitable for immunotherapy are those with the smallest tumor load and are therefore patients in whom risks potentially associated with immunotherapy are more difficult to justify, whereas patients who are most readily available and most need experimental therapy are often those least likely to benefit from the therapy and least likely to yield useful information.

In regard to patient selection it should also be mentioned that, although much of the evidence implicating immunologic factors in neoplasia in animals is derived from studies of viral leukemias, the overwhelming evidence for the existence of TSA in man is, partly for technical reasons, derived from studies of nonhematologic solid tumors. Therefore, in addition to hematologic malignancies, which are certainly valid targets for clinical immunotherapy trials, more investigative attention is indicated for immunotherapy of solid, demonstrably antigenic tumors, especially those which are amenable to surgery but which have a high and predictable incidence of subsequent recurrence.

Both ethical and scientific considerations dictate that attempts at immunotherapy of cancer in man should not compete with other forms of therapy which have a high probability of success. For example, immunotherapy without any other form of therapy should obviously not be attempted in a patient with a localized lymphoma susceptible to radiotherapeutic cure or in a patient with a disseminated lymphoma that is likely to respond to chemotherapy, which in turn would render the patient a more ideal candidate for immunotherapy. In short, immunotherapy should be performed not as a sole form of therapy but as an adjunct to, and in the context of, other potentially effective modes of therapy. In fact, since the patients who are most appropriate for immunotherapy are also most appropriate, on theoretical grounds, for adjuvant chemotherapy, the possibility might be considered of a study comparing adjuvant chemotherapy and immunotherapy in selected patient populations.

In conclusion, developments in tumor immunology in animals and man suggest that, concurrent with appropriate studies in animal models, exploratory attempts are warranted at tumor immunotherapy in man, especially in patients whose tumor load has been reduced by other modes of therapy. *Realistically, perhaps the aim of immunotherapy attempts should initially not be to cure a tumor but rather to prolong a remission induced by other forms of therapy.* The prerequisites for such limited success could then be determined and applied for optimal immunotherapeutic efficacy. All *in vivo* studies should, if possible, be accompanied by correlative immunologic studies *in vitro*, so as to assess the various kinds of immunologic responses elicited by tumor or by unrelated antigens and to determine how the responses are affected by the clinical course of the disease, by conventional therapy and by immunotherapeutic manipulations. Data thus obtained should ultimately lend rational direction to the design of scientifically and therapeutically better approaches to the immunotherapy of cancer in man.

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GUEST EDITORIAL

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Immunotherapy of Cancer in Animals: Models or Muddles?[4]

Immunotherapy, both experimental and clinical, is now fashionable in cancer research. Fashions frequently achieve popularity for irrelevant or inadequate reasons, and they often fade from favor for inappropriate reasons. The former is probably true for immunotherapy. We hope that the latter fate can be forestalled. To that end, we will attempt to answer the following questions: What is immunotherapy? Are animal systems valid models for clinical immunotherapy? What are the imperfections of animal models? How do animal models relate to clinical immunotherapy trials? At the present time, what are the appropriate directions for animal studies?

What is Immunotherapy?[6]

We define immunotherapy as any manipulation that augments the ability of a tumor-rejection immune response to inhibit the growth or spread of an existing tumor. Thus treatments initiated prior to the existence of a tumor are not included, though they may influence the process. The augmentation of tumor-rejection immunity is most directly documented by the development of a greater ability to reject a tumor after therapy than was present before treatment. Local processes may be involved, but the immunity is ultimately systemic and is specifically directed against antigens detectable on tumor cells but not on normal cells. Care must be taken to avoid the "illogism:"

Agent A stimulates immune responses to antigens X, Y, and Z;
Agent A inhibits tumor growth;
ergo, Agent A is an immunotherapeutic agent.

What Does Immunotherapy Have to Offer?

The goal of any cancer therapy is simple—to eliminate all cancer cells in the patient, with as little adverse effect as possible. To accomplish this, the hypothetical ideal treatment would have several characteristics: *strength*—the ability to destroy a relatively large mass of tumor tissue; *specificity*—the ability to destroy malignant cells with little or no damage to normal cells; *systemicity*—the ability to perform discriminative and destructive functions wherever malignant cells may be in the body, regardless of the therapist's ignorance of their presence or distribution. Since we lack an ideal modality, it would be desirable to combine several treatments to achieve complementation of their therapeutic benefits. Unfortunately, the current triad of "conventional" modalities (surgery, radiotherapy, and chemotherapy) all have the same skew to their spectrum of characteristics. They possess strength, but lack adequate specificity or systemicity. The only established treatment with those latter attributes is hormone therapy, which is effective against only a limited number of cancers and is exceedingly weak, because it has no direct cytotoxic effects but operates solely through alteration of cell-cycle kinetics.

Immunotherapy (which cannot now be considered conventional therapy, despite its current vogue) offers the theoretical potential of a systemic treatment with a high degree of specificity and, therefore, low toxicity. In addition, immunologic memory may contribute to post-treatment immunosurveillance against recurrence. These features should constitute the justification for considering immunotherapy as a new weapon in the oncologist's armamentarium. Popularity on other grounds is peripheral to the central issue. "Immunotherapy" which lacks *either* specificity or systemicity has no advantage over simpler, conventional treatments. Nonspecific mechanisms would risk significant damage of normal tissue; this is a major limitation of current therapies. Immunotherapy which is effective only at the site of administration would necessitate treatment at every tumor focus. Currently, the major mode of failure of cancer treatment is our inability to localize minimal, residual tumor, whether it is near the primary lesion or disseminated to the viscera.

Limitations of Immunotherapy

A major limitation of tumor inhibition via tumor-rejection immunity is the relative weakness of the response. At present, it appears that immunotherapy should be restricted to those situations which involve a minimal total tumor mass, such as the residuum that survives optimum conventional treatment. The combination of immunotherapy with other modalities does not necessarily require that the immunotherapeutic maneuvers should be withheld until the failure of the primary treatment is distressingly evident, or even until completion of the conventional treatment. Initiation of immunotherapy prior to surgery, radiotherapy, or chemotherapy, with continuation after the cytoreductive treatment, may facilitate effective immunologic maintenance of remission.

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[6] We will not list the various types of immunotherapy protocols because they have been reviewed elsewhere (*1–4*), nor will we evaluate any specific animal models.

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Weakness of tumor-rejection immunity appears to be universal and insurmountable. Even in experimental situations, an immunity which is effective against a small amount of tumor tissue can be overwhelmed by the presence of a larger tumor burden (*5, 6*). This does not mean that the immune system is intrinsically limited in its ability to destroy malignant tissue. Large masses of tumor tissue can be effectively rejected if they possess the proper antigens (*7*). So the weakness of immunotherapy is due to the nature of cancer-related antigens, not to an incompetence of the immune machinery per se. The elusiveness of tumor-rejection antigens is further compounded by the fact that neoplastic progression may permit tumors to become even less antigenic than they were at the outset (*8, 9*). Thus immunotherapy shares with drug and hormone therapy the problem that resistant variants may arise and become predominant through selective inhibition of sensitive cell populations.

Are Animal Systems Valid Models for Clinical Immunotherapy?

To answer that question, we need to consider what a model is. Webster defines a model as "a description or analogy used to help visualize something that cannot be directly observed," or "a miniature representation of something" (*10*). Thus a model is a substitute for something but is not equivalent to its object. A model differs from its object in some respect(s). A commonsense example illustrates that there can be many kinds of models of the same object. An aircraft may be modeled by a construction mock-up, which reproduces most morphlogic details, including size, but is largely nonfunctional; by a radio-controlled recreational model, which differs in size and detailed morphology, but which can fly; by a wind tunnel model, a proportionately scaled-down replica of the exterior, able to faithfully reproduce all aerodynamic features of the real plane, but wholly incapable of flight; or by a variety of other "representations," each with a unique set of differences from the object. Although each such model presents valid information about the object, each is unable to represent certain other aspects of the aircraft. Thus any model is intrinisically an imperfect or incomplete representation of its object.

In that sense, animal tumor systems can be used as models for immunotherapy in humans. Although they are imperfect representations, the discrepancies between animal and human immunotherapy may be minimized by careful selection of a tumor–host system and by proper experimental design. Some discrepancies will be irreducible, but they should be identified as carefully as possible and must always influence interpretation and extrapolation of results.

What are Imperfections of Animal Models?

Table 1 lists some of the discrepancies between animal models of immunotherapy and the human cancers they purport to represent. None of these differences is universal among animal models, yet no model is free from such problems. Basically, animal models incorporate features of convenience for the experimentalist, a luxury not available to the clinician. Animal tumors are commonly implanted into sites not representative of the natural history of the cancer (e.g., beneath the skin, although skin lesions are not a major problem in humans). Successful models may depend on syngeneic animals as a source of tumor cells for antigen or as a source of sensitized cells, whereas for humans the supply of such materials is limited to the patient's own tissues. Even the most artificial model has the potential to produce new information about the immunobiology of cancer. However, if preclinical investigations are to generate guidelines for the efficient design of appropriate clinical studies, every effort should be directed toward reduction of the discrepancies.

TABLE 1.—*Common discrepancies between animal models of immunotherapy and human neoplasia*

Common characteristics of animal models	Relevant features of human neoplasia
Biologic features of tumors	
Transplanted	Primary, autochthonous
Short latent period	Long latent period
Rapid growth	Slow growth
High growth fraction	Low growth fraction
Sarcomas and leukemias predominate	Carcinomas predominate
Advanced stage of neoplastic progression	Relatively early in neoplastic progression
High antigenicity	Low antigenicity
Infrequent metastases, usually regional	Metastases common, frequently visceral
May carry passenger viruses	Viruses uncommon
Host characteristics	
Young adult	Aged or early childhood
Healthy	Frequently debilitated
Inbred	Outbred
Small body size	Large body size
Short life-span	Long life-span

Perhaps the greatest problem in the field of experimental immunotherapy is the paucity of evidence to establish the general applicability of "principles" developed in animal studies. *Effective* immunotherapy models (tumor–host systems, procedures, and agents) tend to be studied exclusively in a single laboratory, which reminds one of the state of viral oncology 30 years ago. The value of such unilateral investigations can only really be appreciated as observations are confirmed in other laboratories. To this end, there is a serious need for both investigators and editors to maintain a balanced presentation of results, *including negative studies and failure to confirm results of other studies.*

A second basis on which to support the general applicability of experimental models is to avoid reliance on a single tumor line or primary tumor system. Comparative studies in several tumor models will identify results that are peculiar to a single system and will confirm observations with general or clinical applicability. A panel of models should represent a spectrum of tumors in terms of histology and etiology, as well as host species. This will minimize conclusions based on model idiosyncrasies. Another criterion of model selection involves the relative merits of primary tumor as compared with long-transplanted tumor lines. The transplanted lines have the advantage of stability, reproducibility, and a fund of information concerning biologic behavior. In contrast, studies which involve primary tumors, especially in outbred hosts, more closely approximate the variability and unpredictability characteristic of human neoplasia, as well as the features of neoplastic progression, immune surveillance, and spread which may be unique to in situ primary cancer. The most comparable situation would be the study of naturally occurring tumors in the hosts of origin, but that is usually impractical unless it is based on extensive preliminary work with transplanted tumors. At the very least, immunotherapy must be studied in syngeneic systems.

The experience in other areas of oncology has identified two pitfalls to be avoided in developing animal models of immunotherapy. For many years, experimental models used for cancer chemotherapy screening were selected primarily for their convenience in the laboratory (rapid growth and high growth fraction). Drugs selected in such systems have been highly effective against the few human tumors that shared those features but are less effective against the more common, slowly growing human cancers. Similarly, bioassays for carcinogenicity have been efficient in confirming the oncogenic potential of agents under suspicion due to human epidemiologic evidence but have been less valuable in identifying unanticipated hazards to humans.

How do Studies in Animal Models Relate to Clinical Immunotherapy Trials?

Animal tumor models are substitutes for the real thing—human cancer. Therapeutic trials in human cancer patients and studies in animal tumor models are not mutually exclusive, nor should they be antagonistic. Wherever and whenever important questions about management of human cancer can be answered by clinical experimentation or trials, that should be done. Any treatment that consistently and significantly prolongs survival of human cancer patients should be studied in patients and not in animals. Unfortunately, trials of immunotherapy of human cancer have not produced such clear-cut benefits. A large number of agents and protocols have been tested, but effects have been weak, rare, local, or unpredictable. Usually, the approach has been empirically selected from a variety of possibilities. Anecdotal reports have tended to emphasize the rare, dramatic "responses;" it is not clear whether such results have been therapeutic or spontaneous. Trials with retrospective controls suggest that current treatment, including immunotherapy, is better than earlier methods, but they do not tell whether immunotherapy is the only, or the relevant, improvement. Such studies certainly permit the conclusion that immunotherapy, as tried to date, will not be a panacea. Randomized, controlled clinical trials of immunotherapy have been rare. Although they represent the most important, definitive means of establishing improved modes of therapy, they should be the capstone of the evaluation procedures, not the inital screen.

The numbers and types of questions that can be answered by controlled clinical trials are limited. Those limitations are the very area in which animal experimentation offers significant advantages. Any clinical trial, controlled or otherwise, *should* be limited by ethical considerations, ultimately in the form of an investigational new drug or clinical investigation committee approvals. New treatments must be tested initially in patients with advanced disease, and then only in combination with "best available" conventional therapy. However, animal studies permit new approaches to be tested individually as primary treatments and to be compared with the results of withholding all therapy. In animals, it is possible to study large numbers of subjects, and thereby attain greater sensitivity in the detection of therapeutic benefits, better control of experimental variables, and greater capacity to evaluate numerous variables. Assessment time in animal models is short, thus there is less "turn-around" time for confirmation and refinement of the study. Overall, these features establish animal models as an appropriate first level for evaluating new therapies. They can provide direction in the selection of agents and procedures for clinical testing and can help to refine the questions that must ultimately be answered by human experimentation. Thus studies in animal tumor models and therapeutic trials in human cancer patients are complementary and interdependent. The limitations of clinical experimentation and problems of patient care define the goals and relevance of preclinical, animal model studies; the latter, in turn, can focus the efforts expended in human research.

Given the interrelationship of animal and human experimentation, one is confronted with the problem of extrapolating results in animals to the clinical setting. We feel that there are four approaches to the solution of that problem: 1) General relevance of animal results should be established by application in several animal models, representing different tumors and species. Idiosyncratic behavior of systems and agents should be revealed by this strategy. 2) General relevance is further supported by confirmation of results in different laboratories. 3) An understanding of the basic mechanisms by which immune stimulants inhibit tumors (and why they fail in some situations) may assist in the prediction of the clinical value of certain agents or in the design of new ones. 4) Finally, principles of immunotherapy derived from animal models should not be adopted as the basis for routine clinical therapy without confirmation in controlled clinical trials.

What are Appropriate Directions for Animal Studies of Immunotherapy?

Many of the immediate needs in the area of experimental immunotherapy have already been identified in the preceding discussion. Successful animal models need to be confirmed in other laboratories and unsuccessful work should be communicated. Working models should be modified to more faithfully represent the features of human neoplasia. Beneficial treatments should be evaluated in several animal tumor systems. Those treatments which have general applicability should be studied for their mechanisms of action. This would identify those aspects of the immune response which correlate with curative procedures. Furthermore, such analysis should include comparisons of effective and ineffective procedures and responsive and unresponsive models. Such data would develop some basis for *predicting* the efficacy of a given procedure in a given clinical situation. Studies of mechanisms of action should be supported by in vitro analysis of immune responses to identify those tests which may be useful predictors or monitors of the in vivo benefit of immunotherapy. There is a need to identify agents and procedures with potential for synergistic action and to define which types of immunotherapy will work in combination with the more conventional modalities. One approach would be to identify chemical and biological characteristics of immune stimulants and of tumor antigen preparations that contribute to tumor inhibition in vivo. Little is known about the permanence of immunotherapeutic tumor regressions (*11*) or how to avoid late recurrence in individuals "cured" by immunologic means. Finally, with the development of meaningful animal models, immunotherapeutic procedures (immune stimulants, tumor antigen preparations, and administration protocols) can be compared for their relative effectiveness.

A new generation of animal models of cancer immunotherapy is needed, as is confirmation of much work in existing models. This challenge has been well put in the following exchange (*12*).

> "When BCG trials were started in man . . . the rationale seemed to be that it was permissible to attempt to benefit some patients and to ignore the likelihood that others would be harmed . . . The necessary background information should have been a pre-condition for the initiation of trials with living BCG in man." (D. W. Weiss)
>
> "I would press onto laboratory scientists the fact that when human disease is bad, as in acute myeloid leukemia and melanoma, the clinician and the clinical investigator feels the pressure of the bad prognosis. He is not willing to stand around and wait for the animal models to be perfected before beginning carefully conceived efforts to use the best ideas in therapeutic trials." (R. A. Good)

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ADDENDUM

Our Editorial inferred that the "fashion" of immunotherapy might fade for inappropriate reasons. Unfortunately, this prediction has already been realized (*1, 2*). Despite extensive clinical trials, tumor immunotherapy has contributed little of lasting benefit to patient management (*3–5*). The potential reasons for failure are manifold, but few clinical trials have been squarely based on successful protocols in animal systems (*2, 6*). The current fashion in immunotherapy is to criticize the "nonrelevance" of some models (*1, 3–5, 7*). We strongly support the call of recent reviewers for additional animal models of immunotherapy (*3–5, 8–10*). The models should possess at least some of the attributes representative of human cancer, such as naturally occurring tumor, low immunogenicity, or spontaneous metastases. The treatment protocols should also include some actual patient treatment regimens, including immunotherapy after cytoreductive manipulations. In our desire to produce clinical surrogate models, we must not sacrifice experimental feasibility. An animal model, which involves treatment (*4*) of residual, disseminated (*3*) tumor after conventional treatment (*11*) of a naturally occurring (*7*), autochthonous (*10*) carcinoma of elderly (*7*), large (*12*), noninbred (*12*) animals would be as cumbersome for experimental studies as are clinical trials (*4*).

The goals of experimental immunotherapy are to identify effective procedures (screening) and to understand the mechanisms of those procedures (analytical), with a view to the design of better methods. Clinical trials can contribute to those ends, but they do so inefficiently due to the numerous, well-recognized limitations to clinical experimentation. The raison d'être of animal models for immunotherapy is to approach those same goals more thoroughly and more efficiently than is possible for human studies. The assumption that an ideal animal model predictive for human cancer immunotherapy will be available may be as dangerous as the assumption that there is a single best animal tumor for biochemical (*13*) or chemotherapy studies (*14*). The clinical "relevance" of models, agents, and protocols can only be documented retrospectively as early successes emerge from patient trials as mentioned in the Editorial. The major contribution of animal models will be their use in unraveling the mechanisms of successful immunotherapy. The ultimate value of all conclusions in experimental immunotherapy will rest upon independent corroboration in a diversity of animal models (*5*).

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GUEST EDITORIAL

Herbert J. Rapp[1]

JNCI 59:1577–1579, 1977

Of Cavies and Men: The Propriety and Rationality of "Cancer Immunotherapy"

Immunotherapy of human cancer is at an early stage of development and its clinical applicability is controversial (*1*, *2*).[2] Results of animal studies (*3*, *4*), however, suggest that immunotherapy can be applied ethically and optimistically to the treatment of some cancer patients. Cancer immunotherapy will be defined, its mode of action will be discussed, and suggestions, based on a cavian model and a naturally occurring bovine cancer, will be made about the types of cancer patients who might benefit from immunotherapy. The guinea pig model has permitted the development of immunotherapeutic methods that may be effective at a time when microscopic lymph node metastases are present. The naturally occurring disease, bovine "cancer eye," may provide a means of testing the results of model studies under field conditions. Clinical trials of cancer immunotherapy have been and probably will continue to be designed with little or no guidance from animal studies. Differences between animal models of malignant disease and human cancer justify the argument that protocols for the immunotherapy of human cancer based on clinical judgment alone are just as valid as those based on a combination of clinical judgment and the results of animal model studies. The main point of this article is the suggestion that clinical judgment alone may no longer provide sufficient justification for the establishment of appropriate trials of human cancer immunotherapy. Results of studies with naturally occurring animal cancers may guide the clinical practice of cancer immunotherapy onto paths that these studies predict will lead to success.

Regression of human neoplasms following deliberate treatments or accidental events that might be construed to be immunologic have been reported since the latter part of the 19th century. Reports of cancer regression following chance infections such as erysipelas inspired Coley (*5*) to treat cancer patients with bacterial toxins. His clinical studies spanned the years 1891–1936. Anecdotal records of his "toxin therapy" leave little doubt that occasionally striking and sometimes long-lasting regressions resulted from the treatment (*6*). Gross (*7*) in 1943 published the first unequivocal evidence that experimentally induced murine tumors possessed tumor-specific transplantation antigens. Since that time clinicians have provided clear demonstrations of regression, if not cure, of human tumors by immunologic maneuvers. Klein et al. (*8*) sensitized patients suffering from recurrent, multiple, planar basal cell carcinoma to allergens, such as dinitrochlorobenzene, and then applied the allergen directly to the tumors. Tumors regressed following the development of intratumoral inflammation, but no compelling evidence that tumor-specific host immunity contributed to beneficial results was found. Morton (*9*) and others (*1*) have reported regression of cutaneous lesions of recurrent malignant melanoma following intralesional injection of BCG. In some patients, uninjected as well as injected lesions regressed. A few patients have remained tumor-free for several years. In most instances, however, regressions have been temporary, even though sometimes they appeared to be complete. No animal studies that have dealt adequately with recurrent cutaneous metastases have been reported.

Cancer immunotherapy is the promotion of physiologic resistance against carcinogenesis to: 1) prevent establishment of neoplastic disease, 2) limit its advancement and spread, and 3) eradicate malignant disease. The first of these goals has not yet been accomplished in humans and its achievement at present seems remote. The second goal is more likely to be reached than is the first, and the third has been achieved in a limited sense. Use of the term "cancer immunotherapy" is premature because this implies that we know "immunity" to be the means of cancer regressions that sometimes follow immunologic maneuvers. Claims that the "immune system" mediates cancer regression have come from in vitro studies or other investigations with no obvious relevance to established cancer. Even unequivocal demonstrations that tumor-specific immunity appears during immunologic cancer cures in animals have not provided incontrovertible evidence that the immune system is the mediator of such cures. For example, the development of tumor-specific delayed cutaneous hypersensitivity in guinea pigs was one concomitant of cancer cure by the intralesional injection of living *Mycobacterium bovis* (BCG) or a suitable nonliving mycobacterial preparation. Guinea pigs with tumors treated by

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[2] Terry WD, Windhorst D, eds: Immunotherapy of Cancer: Present Status of Trials in Man. New York: Raven Press. In preparation.

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BCG injections at sites remote from the dermal transplants also developed tumor-specific delayed cutaneous hypersensitivity, but they were not cured (*10*).

Studies with this guinea pig model have yielded information that may be applicable to the design of protocols for the treatment of some human carcinomas. The cavian model consists of a transplantable, syngeneic hepatocellular carcinoma that metastasizes to lymph nodes after intradermal implantation of an ascites form of the tumor. Untreated animals never survive once metastases are detectable. Most animals with these tumors growing in their skin can be cured by a single intralesional injection of BCG cell walls incorporated into mineral oil drops in an aqueous emulsion, provided the treatment is given at or before the time that microscopically detectable regional lymph node metastases develop (*11*). Once lymph node metastasis detectable by palpation develops, the probability of cure by a single intralesional injection of the mycobacterial vaccine approaches zero. The requirements for successful "immunotherapy" of guinea pigs with this tumor growing in their skin are: 1) treatment before the development of palpable metastasis and 2) injection of the BCG cell wall preparation directly into the skin tumor. Treatment of animals by administration of BCG at sites other than the lesion has not yielded consistent cures. Some success has been achieved by paralesional injection of the vaccine, followed by excision of the dermal tumor. 3) Treatment only once. Multiple treatments offer no apparent advantage with the guinea pig model. 4) Saturation of all visible parts of the tumor with the vaccine. 5) Injection of an adequate dose of vaccine. Achievement of cure rates of 80–100% required about 1 mg of cell walls. 6) Capability of animals to have an immunologic response to BCG antigens.

Additional results with the guinea pig model indicated that tumor immunity was unable to cope with "minimal residual disease" remaining after radical surgery (*12*). Guinea pigs with a growing, transplanted, intradermal carcinoma, palpable draining lymph node metastases, and microscopic (nonpalpable) metastases in the next lymph nodes of the chain were treated by intratumoral injection of BCG into the dermal transplant. A week after BCG inoculation, the injected tumor and the draining lymph nodes were excised, leaving minimal residual disease in the form of microscopic metastases in the next lymph nodes of the chain. Eventually, these animals died with large, palpable lymph node metastases at several sites throughout the body. Excision of growing primary transplants, draining lymph nodes, and the next group of lymph nodes in the chain without administration of BCG cured some animals with or without palpable metastases in the draining lymph nodes. Tumor immunity developed in animals cured by radical surgery performed at a time when the draining lymph nodes were palpable but did not develop in animals treated surgically at an earlier stage in the disease when no palpable metastases were detected. In these experiments, tumor immunity was assessed by the ability of animals to reject an intradermal transplant of tumor at a site remote from the original challenge site (contralateral challenge). A single intralesional injection of BCG without surgery was effective therapy only when no metastases existed or when spread was limited to microscopic (nonpalpable) draining lymph node metastases. Animals cured by intralesional BCG developed the ability to reject a contralateral challenge. Not only was intratumorally administered BCG ineffective against palpable metastases, but also it had no influence on the results of subsequent radical surgery.

Even the demonstration that animals with growing transplants and microscopic regional metastases could be cured by the passive transfer (adoptive immunization) of lymphoid cells from healthy donors that were hyperimmunized with a mixture of BCG and tumor cells (*13*) did not prove that "sensitized" lymphoid cells mediated cures in animals treated by intralesional BCG.

Active specific immunotherapy in the form of intradermal inoculations of living tumor cells admixed with BCG was effective therapy only if given well before the development of microscopic metastasis (*14*). Experiments are in progress to determine whether active, specific immunotherapy will be effective against minimal residual disease. Should specific immunotherapy be effective under that condition, support for some clinical trials will be provided.

The guinea pig model, like all models, is limited in its applicability to human cancer. Recently, however, the guinea pig model was used in the design of studies of naturally occurring, metastatic, bovine ocular squamous cell carcinoma (*15, 16*). In those studies, 70% of cattle with primary cancer and without palpable metastases treated by intralesional injection of BCG cell wall vaccine have had either regression or arrest of disease. Some of these animals have been free of visible tumors for more than 2 years. All untreated animals have progressive malignant disease.

That tumor regression can be induced by apparently immunologic methods is remarkable, because immunotherapy or vaccine therapy for infectious diseases is effective prophylactically but rarely after disease symptoms appear. The hope that the treatment of minimal residual malignant disease would be tantamount to immunoprophylaxis received support neither from our guinea pig studies nor from relevant clinical trials that had been in progress long enough to draw valid conclusions (*2*).[2] Logically then, patients with untreated primary stage I disease are the prime, perhaps at present the only, candidates for immunotherapy.

Results of studies with guinea pigs and cattle lead me to make the following suggestions for future clinical trials of immunotherapy: 1) Use BCG cell walls at a concentration of 1 mg per ml incorporated into mineral oil droplets emulsified in salt solution (*11*). The mineral oil accounts for only 3% or less of the emulsion. Nonliving BCG is recommended to avoid the potential of disseminated mycobacterial infection. 2) Treat patients with previously untreated primary cancer and with at most microscopic metastases in the draining lymph nodes. 3) Administer the BCG cell wall vaccine intratumorally. 4) Treat only once and in such a way as to saturate all visible tumor with the aqueous emulsion of mineral oil containing BCG cell walls. 5) Perform only those additional cancer therapies that are ethically required but not until at least 2 weeks after inoculation of BCG cell wall vaccine. 6) Be prepared to deal with possible untoward side effects. 7) Determine the "immunologic status" of patients before, during, and after treatment.

These suggestions impose severe but surmountable barriers to the initiation of clinical trials of cancer immunotherapy based on relevant animal studies. The following cancers seem particularly suited to immunotherapy trials: 1) stage I inoperable bronchogenic cancer occurring at or near the carina. 2) stage I squamous cell cancer of the penis in patients who refuse resection. 3) stage I inoperable cancer of the anus. 4) stage I cancer of the head in which surgery would cause unacceptable mutilation. 5) stage I, Clark's level 4, malignant melanoma. A trial of this disease is in progress at the National Cancer Institute, but living BCG is being used rather than cell wall vaccine. 6) Any other stage I malignancy in which the best clinical judgment predicts lack of success by or contraindicates the use of currently accepted treatments.

Success with any or all of these cancers ultimately might permit application of immunotherapy to patients with cancer curable by currently accepted treatments.

Robert Burns, in his poem *To a Mouse*, opened our minds with the line "The best-laid schemes o' mice an' men Gang aft agley." The best planned clinical protocols of cancer immunotherapy may also go awry unless the plans are based on animal studies that are as close to clinical reality as possible.

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ADDENDUM

It was suggested in the Guest Editorial entitled *Of Cavies and Men: The Propriety and Rationality of "Cancer Immunotherapy"* that, according to knowledge then available, treatment of human cancer by immunologic maneuvers should be used at an early stage of disease and only when other forms of treatment are inappropriate. It was pointed out that some success had been achieved by the intralesional injection of mycobacterial vaccines in the eradication of malignant disease but immunotherapeutic maneuvers had not yet been found to prevent carcinogenesis nor to limit the growth and spread of neoplastic disease. Recent information derived from animal studies has suggested that active specific immunization with the aid of appropriate immunologic adjuvants may be effective in eliminating malignant disease remaining after surgery (*1*). This success suggests that attempts should be made to limit the growth and spread of neoplastic disease in humans by active specific immunization following surgery. In the animal experiments, a transplanted syngeneic hepatoma growing in the skin of each of several guinea pigs was excised, and microscopically detectable lymph node metastasis remaining after surgery was eradicated by a single intradermal injection of living hepatoma cells mixed with emulsified BCG cell walls. Treatment with the adjuvant and tumor cell mixture was effective when given near or remote from the surgical site. Mixtures of living BCG and tumor cells eliminated lymph node metastasis when given near the surgical site but failed to do so when given on the side contralateral to the site of tumor excision. Tumor cells used for immunization of human patients would have to be derived from tissue removed by the surgeon and the cells injected with emulsified BCG cell walls would have to be treated (perhaps by X-irradiation) to render them incapable of replication. Naturally occurring animal cancers are being studied to test the feasibility of active specific immunization in the treatment of malignant disease remaining after surgery.

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GUEST EDITORIAL

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JNCI 60:1191–1194, 1978

Interferons and Cancer[2]

Although interferons were discovered in 1957 (*1*), we do not yet fully understand their production, mechanism of action, biologic role, or therapeutic potential. Originally described as antiviral agents, interferons have other activities which include regulation of cell growth (*2*) and regulation of the immune response (*3*). Several recent findings have made interferons of current special interest to oncologists. Through the persistent effort of Dr. Kari Cantell, Central Public Health Laboratory, Helsinki, Finland, an amount of human leukocyte interferon sufficient for meaningful clinical studies of the effects of interferon on a few diseases has finally become available. Employing this interferon, a group at Stanford University, Stanford, California, under the direction of Dr. Thomas Merigan, has obtained evidence suggesting that human interferon may be useful in the treatment of chronic hepatitis B virus infection (*4*) and has found that interferon treatment of herpes zoster complicating leukemias and lymphomas has led to more rapid resolution of lesions and prevention of systemic effects (*5*). In addition, preliminary results from the laboratory of Dr. Hans Strander (Karolinska Institutet, Stockholm, Sweden), who has been treating 28 osteogenic sarcoma patients with Cantell's human interferon, suggest that the group receiving interferon has a lower rate of metastasis than does a concurrent control group (*6*). Strander's studies, although not yet involving sufficient numbers of patients to be highly significant, appear to warrant serious consideration of human interferon as an antitumor agent; animal studies supply strong evidence that in some situations interferons are effective in inhibiting tumor growth (*7*).

Interferons are proteins. Their production by animal cells can be induced by various substances (*1*), and they in turn inhibit a wide spectrum of RNA and DNA viruses by inducing an antiviral state. However, interferons are generally animal species-specific in their range of antiviral activity; only human interferons have been shown to be effective in treatment of human cells, although it is certainly possible that some animal interferons will prove active.

INTERFERON PRODUCTION

Interferon production is an induced activity. Under normal conditions animals produce interferons as one of a series of responses to virus infections, and interferons seem to be important in recovery from some primary virus infections (*7*). Lymphocyte interferons are lymphokines, inasmuch as their production may be induced by exposing macrophages and T-lymphocytes to mitogens or antigens (*8*). Interferons may also be induced in tissue cultures by double-stranded RNA forms such as polyriboinosinic-polyribocytidylic acid (poly I · poly C) and in animals by, for example, various intracellular parasites (bacteria, metazoa, protozoa), polymers, endotoxins, or double-stranded RNA forms (*9*).

When exposed to an interferon inducer, cells in culture make an interferon messenger RNA, which may be translated in cell-free systems or in frog oocytes (*10, 11*). The amount of interferon produced by cells is proportional to the amount of interferon messenger RNA induced (*12*). The amount of messenger RNA may be increased by a superinduction procedure which involves treatment of cells with inhibitors of RNA and protein synthesis, after exposure to an inducer but before collection of interferon begins (*13*). The superinduction procedure appears to prolong the half-life of the interferon messenger RNA (*12*). The production of interferon presents the cell biologist with an interesting induction system in which negative feedback controls operate and an easily characterized protein is made.

ANTIVIRAL ACTIVITY OF INTERFERONS

Although the mechanism of action of interferon is not yet known, it seems to be the result of a complex series of events (*14*). Interferons bind on the cell surface to a specific receptor which seems to contain both ganglioside and peptide components [(*15*); Kohn LD:

ABBREVIATION USED: MuLV = murine leukemia virus.

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

Unpublished data]. Binding of interferon to a cell surface receptor does not necessarily result in the development of antiviral activity, but in sensitive cells, i.e., those with the proper effector apparatus, binding causes chemical, morphologic, physical, and immunologic alterations in the plasma membranes (*16–18*). These alterations, possibly through a mechanism involving cyclic AMP (*19, 20*), seem to result in the production of an enzyme precursor which when activated can produce an intracellular antiviral substance (*21*). This enzyme is a protein, the production of which probably requires the synthesis of a new species of messenger RNA (*22*).

After an interferon-treated cell is infected with a virus, the production of a viral product, most likely a double-stranded form of viral RNA, seems to trigger the activation of the enzyme precursor (*23, 24*). The activation may involve phosphorylation of the precursor by a kinase also activated by a viral double-stranded RNA. The antiviral substance formed in cell-free extracts is a recently described adenosine oligomer with an unusual 2′,5′ linkage (Kerr IM, Ball LA: Personal communication). This antiviral substance may inhibit virus replication by several mechanisms, which possibly include inhibition of virus-directed transcription, or translation, or ribonucleolytic cleavage of viral messenger RNA.

SITE OF ANTITUMOR ACTIVITY OF INTERFERONS

Several hypotheses have been advanced to attempt to explain a putative antitumor activity of interferon. These include inhibition of tumor virus replication and cell transformation by virus and inhibition of tumor growth, directly or indirectly, through primary effects on the immune system.

Interferon was thought to inhibit tumor viruses through the same mechanism as that involved in the inhibition of other viruses (*14*). For the inhibition of both the DNA and RNA tumor viruses studied most completely, however, the mechanism of interferon action seems to have unusual features. For simian virus 40, the accumulation of early viral messenger RNA is inhibited (*25*), whereas in the best studied of other systems, early viral messenger RNA is not reduced by interferon treatment (*26*). Even more surprising were the findings on interferon inhibition of MuLV replication, a system in which interferon treatment does not seem to inhibit MuLV-directed protein or RNA synthesis (*27, 28*). Instead, defective virus with markedly reduced infectivity is synthesized (*29–31*). In some systems, the MuLV produced in interferon-treated cells is so defective that it cannot bud from the cell surface (*32, 33*). At least two abnormalities have been found in the defective MuLV released from interferon-treated cells: a large glycoprotein, possibly a precursor to the viral gp69/71 in the viral membrane (*34*), and a defective p30 reverse transcriptase complex with decreased enzyme activity (Bandyopadhyay AK: Personal communication).

Whatever the mechanism, however, interferon treatment results in a marked decrease in the production of infectious virus and in cell transformation by virus (*35*). Interferon may thus prevent virus recruitment of new cells into the transformed state, which may explain the repeated observations in many systems that interferon treatment inhibits induction of tumors in mice by oncogenic viruses (*2*). Interferon treatment also inhibits the growth of established virus-induced mouse neoplasms and has been successfully employed to inhibit transplantable tumors and chemically induced neoplasms in mice (*2*). Less success was usually attained in the treatment of solid transplantable tumors than in treatment of ascites tumors; however, interferon treatment inhibited the development of both the subcutaneous nodules of Lewis lung carcinoma at the site of transplantation and the development of pulmonary metastases from the transplant (*36*). Interferon administration reduced the incidence of fibrosarcomas and lung adenomas induced in CF_1 mice by 3-methylcholanthrene (*37*). These transplantable or induced tumors are not obviously due to viruses. Their inhibition by interferon suggested that interferon treatment might have a direct effect on tumor growth.

The inhibition of growth of tumors which apparently are not virus induced gave rise to speculation that interferon might be a growth control regulator and that the effects on tumors are caused by rapid tumor growth. If interferon does regulate the growth of cells, interferon treatment should show some effects on normal (nontumor) cells. In some studies, this has proved to be so, and in vitro interferon inhibited cell DNA synthesis and replication (*38*). Interferon treatment inhibited the regeneration of the liver in partial hepatectomized mice (*39*). Newborn C3H or Swiss mice treated with high concentrations of partially purified interferon exhibited weight loss and diffuse hepatic cell degeneration (*40*). Similarly, in a newborn child treated with human interferon for congenital cytomegalovirus infection, significant weight loss was recorded before the therapy was halted (*41*).

A third possible site for interferon-induced inhibition of tumor growth is the immune system. In both in vivo and in vitro studies, interferon treatment inhibited the antibody response (*42*). This effect of interferon was dose-dependent and could not be separated from the antiviral activity of the interferon preparations. Interferon also inhibited cellular immune responses and delayed-type hypersensitivity (*43*). Similar studies also showed an inhibition by interferon treatment of in vitro mitogen-stimulated DNA synthesis by T-lymphocytes (*44*). In mice, interferon treatment inhibited the response to allografts (*45*), sensitization to picryl chloride (*43*), and the delayed-type hypersensitivity response to sheep red blood cells (*46*).

Possibly related to studies on the immune system was the finding that interferon treatment inhibited phagocytosis (*47*) in mice and caused several specific alterations in the surfaces of lymphocytes. Interferon-treated lymphocytes had an increased capacity to absorb alloantisera (*17*) and an enhanced expression of surface H-1 antigens (*48*).

Thus interferon treatment might inhibit tumor growth in several ways through primary effects on the immune system. Alterations induced by interferon in the plasma membranes of lymphocytes might be responsible for an increased cytotoxicity. Interferon treatment could also alter the surfaces of tumor cells (as it does in lymphocytes and L1210 cells) to increase the expression of tumor-specific transplantation antigens. The increased phagocytosis induced by interferon treatment could have a role in the inhibition of tumor growth by causing rapid destruction and elimination of tumor cells. Finally, the inhibitory effects on antibody production could cause a decreased level of tumor-protective (blocking) antibodies, thus permitting the immune system to deal more effectively with tumors.

ATTEMPT AT A UNITARY HYPOTHESIS TO EXPLAIN THE VARIOUS ANTITUMOR EFFECTS OF INTERFERON

Many diverse effects of interferon treatment have been discussed so far. These include inhibition of viral RNA and protein synthesis, formation of defective virus particles, inhibition of normal and tumor cell growth, and inhibition of various aspects of the immune response. Can all of these activities be explained by interferon action at a single site? This is a difficult synthesis, but it is possible that changes induced by interferon on the plasma membranes of cells might be common initial sites of action. Antiviral action seems to be started by the binding of interferon to plasma membrane receptor sites and then, in sensitive cells, its affecting activation sites. These steps appear to cause several alterations in the plasma membrane. For RNA tumor viruses, the interferon-induced alterations in the plasma membrane could be responsible, at least in part, for the structural defects in virus produced by these cells. For other viruses, the early events (binding and activation) may be responsible for the intracellular activities that later lead to the inhibition of virus growth.

Alterations in the plasma membrane could also help to explain interferon's inhibitory action on normal and tumor cell growth. The phenomenon of contact inhibition of growth would suggest that events at the cell surface are important in determining the replication rate of cells. Interferon-induced changes in the plasma membrane could signal a cutback in the growth rate of malignant cells.

Finally, many of the effects of interferon on the immune system could be related directly to effects on the plasma membrane. Certainly, as discussed above, most of the effects on the immune system which may be related to the antitumor activity of interferons are membrane-associated events. These include increased phagocytosis, an increase in expression of transplantation antigens, and an increase in cytotoxicity of interferon-treated lymphocytes. Therefore, although it is a very speculative hypothesis, the actions of interferons may be due to a primary effect on the plasma membranes.

WHAT CAN BE DONE?

In spite of the promise of the investigations outlined above, the number of researchers in the field of interferon studies is small, and support for basic research has been modest. There are several reasons for this situation. Because of its great specific activity (one unit has been recently estimated to be less than 10^{-9} mg of protein), interferons have been difficult to obtain in large enough quantities for purification or for a significant number of clinical studies. In addition, assays for interferon are biologic, based on the ability of a preparation to inhibit virus replication; they are time-consuming and relatively inaccurate. Finally, in any one species several substances appear to have the general properties of interferons. For example, human white cell interferon preparations contain several molecular species of interferon (*49*). Interferon produced in human diploid cells has one component that antigenically resembles one of the interferons from white cells. Human T-lymphocytes, stimulated by mitogens or antigens, produce yet another distinct species of interferon. Although all of these interferons induce antiviral activity, it is not clear that they act in the same way.

It is no great wonder, then, that many editors and peer review groups are unenthusiastic about interferon research. Only because interferon had promise as an antiviral agent was it fortuitously included among the drugs to be studied by the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland. For several years, under the excellent supervision of Dr. George Galasso, this program was probably the greatest source of funds for interferon research in this country. In many areas of interferon research, the Antiviral Substances Program was responsible for keeping up some momentum at a time when progress would otherwise have been stalled. At present, however, I believe that the exciting research leads on the molecular biology of interferon production and action, together with the preliminary data, discussed above, on the clinical uses of human interferon, should stimulate cancer researchers with a wide range of interests to consider doing studies in these areas. Also, large-scale bioengineering techniques should be used in the attempt to deal with the problems of interferon production and purification.

Such efforts may lead to solutions of basic problems in the understanding of biologic controls of animal cells. In addition, the leads that Gresser (*2*) and Strander (*6*) have put forth may be confirmed, and interferon could prove to be useful in the therapy of cancer.

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GUEST EDITORIAL

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The Emerging Genetics of RNA Tumor Viruses

IN RECENT MONTHS several discoveries have set the stage for a genetic analysis of RNA tumor viruses. Conditional lethal mutants of avian sarcoma viruses have been isolated and characterized physiologically (*1–8*). They can be assigned to three major categories: 1) those which fail to transform the cell but replicate at the nonpermissive temperature, 2) those which do not replicate but transform under restrictive conditions, and 3) those which have a coordinate lesion affecting both transformation and replication in a nonpermissive environment. Further physiological experimentation will no doubt add to our knowledge of viral gene functions and at the same time will call for genetic studies including tests for recombination and complementation between the mutants.

Several types of nonconditional mutants have also been described. To these belong the various nontransforming (NT) viruses isolated from helper-independent Rous sarcoma virus (RSV) (*9–11*), the replication-defective mutants of Prague strain RSV described by Weiss (*12*), and the alpha form of the Bryan high-titer strain of RSV (*13, 14*). The ease with which RNA tumor virus mutants can be obtained suggests that there will soon be enough of them to allow exhaustive genetic studies. This article considers the likely ramifications of RNA tumor virus genetics and formulates specific hypotheses which are testable and thus may be proved or refuted.

The Viral Genome

The characteristic features of inheritance among RNA tumor viruses must be dictated by the structural peculiarities of the viral genome, its possible interaction with the cell, and its replication and assembly into progeny virus. Since our knowledge of these subjects is fragmentary, sparse facts must be supplemented with assumptions and speculations to arrive at a coherent picture from which genetic hypotheses can be developed. The viral genome is probably the 70S RNA extractable from purified virions (*15*), though genetic contributions from the 7S component have not been ruled out (*16*). The 70S molecule does not seem to have a continuous sugar phosphate backbone but

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appears to consist of 35S subunits linked together by hydrogen bonds (*17–19*). One 70S molecule may contain 3–4 such subunits.[1]

This segmentation of the viral genome must have important genetic consequences and will be a key assumption in the hypotheses to be presented.

The subunits of the 70S RNA are not identical; otherwise, all RNA tumor viruses would be polyploid. The kinetics of viral inactivation by radiation or chemicals speak against polyploidy of the total genome, though they do not rule out the existence of particles in which certain genome segments are present in duplicate or triplicate (*9–11*, *22–24*). A functional specialization of genome segments is also supported by the finding that the 70S RNA of avian sarcoma viruses contains at least 2 size classes of subunits (*25*).

By analogy with other RNA viruses having a segmented genome, the 70S tumor virus RNA in all likelihood does not replicate as a unit. Whatever the mode of replication—via a DNA intermediate or via double-stranded RNA or both—progeny RNA is probably made in 35S segments. The viral RNA found in cells infected with RNA tumor viruses does indeed contain 35S components together with larger and smaller viral RNA molecules (*26*, *27*). The 35S subunits must be assembled into 70S molecules and complete virions by an unknown process. One may distinguish 2 basic mechanisms of assembly: In one, the different kinds of subunits would be packaged at random, with the only limitation being the number fitting into a virus particle; in the other, the combination of subunits would be more specific, avoiding the inclusion of 2 subunits of the same type in one virion. These mechanisms would have different genetic consequences.

RNA tumor virus infection has a requirement for early DNA synthesis and continuous DNA transcription (*28–40*). The DNA synthesized during early infection appears to be genetically specific for the infecting virus (*41*, *42*). It may be the product of reverse transcription from the 70S RNA by an enzyme in the virus (*43*, *44*). This viral DNA may become integrated with host DNA, and progeny RNA would be generated by transcription of this DNA.

Hypotheses

From these facts and speculations it is possible to postulate 3 genetic characteristics of RNA tumor viruses.

1) Defective particles lacking a segment of the total genome should arise spontaneously during replication of RNA tumor viruses. If the assembly of genome segments into virus is random, *partial* diploids or even triploids might be produced.

2) In double infections with genetically distinct RNA tumor viruses, reassortment of genetic markers located on different genome segments should occur.

3) RNA tumor viruses might occasionally contain *cellular* genetic information in the 70S RNA.

Predictions 1 and 2 are made by analogy with influenza and reoviruses, both of which have a segmented genome and consequently segregate defective particles during replication and show genetic reassortment of markers (*45–50*). Prediction 3 assumes the integration of viral into cellular DNA.

Commentary

Spontaneous origin of defective particles: The term "defective particle" is used here in a broad sense encompassing defects of transforming ability as well as replicative capacity. NT viruses have been isolated repeatedly from nonmutagenized, helper-independent stocks of avian sarcoma viruses (*50–53*). Since these agents are always antigenically identical to the virus which they accompany, they are not accidental, extraneous contaminants. Recent experiments indicate that they appear spontaneously in cloned stocks of sarcoma virus, even if the virus is grown in cells free of inducible endogenous avian leukosis viruses (*54*). These

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[1] Other interpretations of the structural data on 70S RNA exist but are considered less likely:

a) The viral RNA does not consist of subunits but is a single molecule. The 35S and 70S components could represent two conformationally different states of the same molecule (*20*).

b) The 70S could be a composite molecule which forms only during extraction and does not occur in the virus. The viral genome would be a 35S molecule with "sticky ends" (*21*). If this were true, one would expect that the 35S components obtained after heating of 70S RNA spontaneously reaggregate, which is not the case.

NT agents may thus constitute one form of defective particle resulting from faulty assembly of genome subunits. NT agents not only occur in sarcoma virus stocks, but are also associated with RNA leukemia viruses. They then fail to transform hematopoietic cells *in vitro*, and, compared to their companion leukemia virus, their leukemogenic potential in the animal is greatly reduced or nondetectable, though they may cause other types of tumors (*55–58*). Other types of defective particles may be represented by Bryan high-titer RSV and murine sarcoma virus, which are unable to produce infectious progeny in the absence of exogenous or endogenous helper agents (*59–63*). An analogous replicative defect has been observed in stocks of avian myeloblastosis virus which contain transforming, *i.e.*, leukemogenic, particles requiring a helper virus for the synthesis of infectious progeny (*64*). Besides NT agents and replication-defective, transforming viruses, RNA tumor virus preparations may harbor other types of defective particles not commonly detectable because they neither transform nor replicate. Defective particles may either contain the standard number of RNA subunits, with perhaps a duplication of one genome segment and corresponding lack of another, or they may actually possess fewer RNA subunits than complete virus. Depending on the physiological conditions prevailing during infection, defective particles may constitute the bulk of the virus yield.

Reassortment of markers: If Bryan high-titer RSV is grown together with Rous-associated virus (RAV), the infectious sarcoma progeny particles are pseudotypes that have acquired the host range marker of the helper virus by phenotypic mixing (*65–67*). No recombinants between the transformation marker of the sarcoma virus and the host range marker of RAV have been described in the literature. The unquestionable importance of phenotypic mixing in the rescue of defective RSV has delayed a search for stable genetic recombination or reassortment of markers. However, there are indications in the literature that recombination or reassortment may occur among RNA tumor viruses. For instance, helper independent sarcoma viruses belonging to subgroups A, B, and C have been isolated from the Prague strain of RSV (*52*). These could have arisen by recombination of the sarcoma virus with Rous-associated viruses of the respective subgroups. Recombinational repair of genetic defects has also been proposed for the alpha form of Bryan high-titer RSV and for endogenous leukosis viruses which can be activated by superinfection of chicken cells with replicating leukosis viruses (*68*, *69*). Genetic reassortment between markers of certain avian tumor viruses is further favored by the results of a current study on mixed infections (*70*). It was found that the transformation marker of a helper independent sarcoma virus could be combined with the host range marker of a leukosis virus in what appeared to be a genetically stable form. The incidence of such viral combination forms was about 50% after several cycles of replication, suggesting a genetic equilibrium between unlinked markers, as may be expected if the corresponding genes are on independently replicating segments of the viral genome. Whether the assembly of genome subunits into progeny particles is entirely random cannot be decided from the available data. Since the number of subunits per particle must be limited, the partial diploids and triploids that might arise from random packaging would inevitably lack certain genome segments. Such defective particles could be difficult to detect. Even if they replicated, the special parental combination of genome segments would appear only in a fraction of the progeny particles.

The failure of the Bryan high-titer RSV to yield helper-independent recombinants after mixed growth with an RAV can only be speculated on. One possible explanation would be nonrandom reassortment of genome segments, perhaps dictated by a specificity in the patterns of hydrogen bonds which hold genome subunits together. Viewed in this context, the defectiveness of Bryan high-titer RSV and murine sarcoma virus may reflect an inability for undergoing genetic reassortment with helper viruses in contrast to helper-independent strains of RSV that may acquire host range markers of the helper virus more readily by genetic reassortment. The preliminary studies mentioned above (*70*) also suggest that not all avian leukosis viruses are able to donate a host range marker to helper-independent RSV or acquire a transformation marker from RSV.

Further restrictions on genetic reassortment may be imposed by the systematic position of the viruses in question. For instance, murine viruses may not be able to interact genetically with avian viruses.

Cellular contributions to RNA tumor virus genetics: One of the striking properties of RNA tumor virus genomes is their variation in size (*25, 71*). Indications are that some leukosis and leukemia viruses as well as the NT agents derived from sarcoma stocks contain less RNA than sarcoma viruses. At least two size classes of subunits derived from the 70S viral RNA can be separated by gel electrophoresis. The larger one, "*a*," has so far been reported only in sarcoma viruses, whereas a smaller one, "*b*," can be found in both sarcoma and leukosis viruses (*25, 72*). This observation suggests that class *a* subunits of sarcoma viruses are somehow related to the ability of the virus to transform fibroblasts. However, it would be naive to assume that all class *a* and all *b* subunits are genetically homologous. Therefore, class *a* subunits may exist which are not linked to transforming ability (*73*). The important point is that viruses containing only class *b* subunits are perfectly viable and that the additional, presumably oncogenic, information which may be present in class *a* is *nonessential* for the perpetuation of the virus. There is thus no reason to assume that the oncogenic information of class *a* subunits is necessarily viral in origin; rather, this information could have arisen from the cell. It may have become part of the virus by being transcribed together with the integrated viral genome. This hypothetical association of oncogenic cellular with replicative viral genome elements does not, however, occur regularly; otherwise, any nontransforming RNA tumor virus growing in a chemically or physically induced sarcoma cell would become sarcomagenic, which is generally not the case but may happen on occasion (*74*). In more general terms, this speculation considers RNA tumor virsuses as potential carriers of cellular genetic information. This transducing activity need not be limited to "oncogenes" but may include other cellular markers as well. Indeed, some older observations on RSV can be interpreted as showing transduction of cellular properties (*75, 76*). A cellular origin of the oncogenic information in RNA tumor viruses may also explain the conspicuous tissue specificity of viral transformation. While transformation by RNA tumor viruses usually shows a requirement for specific target cells, virus replication is remarkably unaffected by the cellular state of differentiation. Replication of tumor viruses can occur in cells that do not become transformed. It is perhaps significant that under these circumstances the oncogenic potential of the virus is often reduced or altogether lost and that NT agents prevail among the progeny.

A complication: Preexistence of viral genetic material in the cell: Many and perhaps all normal cells of chickens and mice (the only species studied extensively) carry genetic information of RNA tumor viruses. This information may be expressed in the form of viral antigens (*77–79*), or in the form of a helper factor that can be activated by superinfection with another RNA tumor virus (*59, 60*). Even cells in which neither viral antigen nor helper factor is detectable can be induced to release RNA tumor viruses by treatment with physical or chemical carcinogens (*80, 81*). This omnipresence of RNA tumor virus genomes must be considered in the design and interpretation of genetic experiments. Obviously endogenous tumor viruses could contribute information to viral progeny. In chicken cells, all endogenous viruses appear to belong to the same subgroup (E) which can be recognized by its specific envelope properties. These and additional tests for markers derived from endogenous viruses will be essential to proper controls and hence to obtaining significant results in genetic studies with RNA tumor viruses.

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ADDENDUM

It is a sobering experience to read one's speculations 6 years later and to realize how misleading instinct and intuition can be, if they select from a collection of fragmentary data only those which fit a preconceived picture. The 1972 Guest Editorial is fundamentally wrong in its ideas about the structure of the oncovirus genome. The complexity of this genome is not that of a 70S single-stranded RNA but corresponds to a 30S molecule (*1–12*). It is neither segmented nor haploid but diploid (*13–17*). Recombination in oncoviruses is not a reassortment of genome subunits but a crossing-over between homologous nucleic acid molecules (*18–20*). We know that the generation of defective particles cannot be explained by the loss of genome segments, but we have few positive facts about the mechanism of genetic deletion in oncoviruses. The only speculative prediction of the Editorial that is now supported by experimental data concerns the origin of transforming genes and the ability of transformation-defective viruses to acquire genetic information from the cell. Sequences closely related to *src*, the transforming gene of avian sarcoma viruses, have been found in normal chicken cells (*21–24*). Certain *td* viruses have been shown to acquire cellular sequences, even to pick up cellular *src* and regain transforming ability for fibroblasts [(*25–27*); Vigne R, Breitman M, Muscovici C, et al: Manuscript in preparation]. The genetics of oncoviruses has become an extremely active and productive field, and much more remains to be learned in this area.

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GUEST EDITORIAL—by Maurice Green and Heschel J. Raskas

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Molecular Hybridization: A Powerful Approach to the Detection of Viral Nucleic Acid Sequences in Human Cancer[1]

IN RECENT years impressive progress has been made in understanding the molecular events during the replication of RNA and DNA tumor viruses and the mechanism of cell transformation by these viruses (*1*). These advances have been possible because the powerful and extraordinarily sensitive tools of molecular hybridization enable the detection and quantitation of viral nucleic acids in productively infected and transformed cells (*2*). In addition, these studies provide the viral nucleic acid reagents and hybridization methodology so that human tumors can be analyzed for viral genetic information (*3*). We describe our current understanding of the mechanism of cell transformation by RNA and DNA tumor viruses and the development and utilization of molecular hybridization approaches for the detection of viral nucleic-acid sequences in human cancer.

Oncogenic Viruses

About 150 of the 600 known animal viruses are oncogenic (table 1). Oncogenic DNA viruses can undergo either of 2 types of interaction with cells: 1) *productive infection*, in which many virus particles are assembled and the cell is eventually killed or 2) *cell transformation*, in which virus does not replicate but the cell is altered morphologically and cell multiplication and macromolecular synthesis are controlled in part by viral genes. Although the DNA tumor viruses, polyoma, simian virus 40 (SV40), and human adenovirus have been used as model systems for the study of virus-induced cell transformation (*1*), only members of the herpesvirus group have been associated with natural cancer in humans, *e.g.*, Burkitt's lymphoma and nasopharyngeal carcinoma.

TABLE 1.—Oncogenic viruses

DNA viruses (~ 50):
  Papilloma: man, dog, cow
  Polyoma: polyoma (murine), SV40 (simian)
  Adenoviruses: 12 of 31 human, 6 simian, 2 avian, 1 bovine
  Herpesviruses: Burkitt's lymphoma (human), Lucké carcinoma (frog), Marek's disease (chicken)
RNA viruses (~ 100):
  Avian leukemia-sarcoma
  Murine leukemia-sarcoma
  Murine mammary tumor
  Leukemia-sarcoma: cat, hamster, rat, guinea pig
  Human milk (?)*
  Human RD-114 (?)†

*Moore *et al.* (*4*).
†McAllister *et al.* (*5*).

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[1] This study was conducted under Public Health Service contract No. PH43-67-692 within the Special Virus Cancer Program of the National Cancer Institute, by Public Health Service grants AI 01725 from the National Institute of Allergy and Infectious Diseases and CA 12560-01 from the National Cancer Institute, and by grant DRG 1156 from the Damon Runyon Cancer Fund. One of the authors (M.G.) is a recipient of Research Career Award 5-K6-AI 4739 from the National Institute of Allergy and Infectious Diseases.

Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

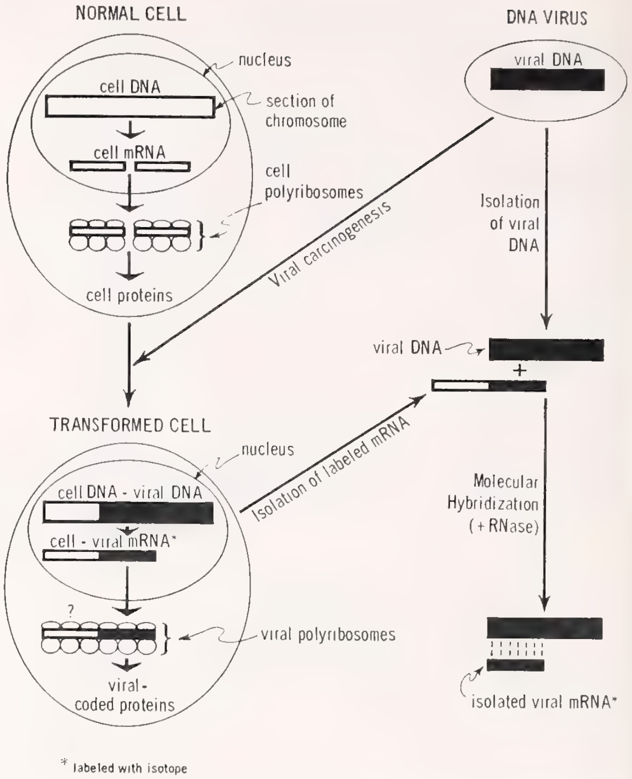
RNA tumor viruses, *e.g.*, sarcoma and leukemia, induce cancer naturally in several animal species. The relationship between virus production and cell transformation is complex, especially since most sarcoma virus preparations contain an excess of leukemia virus. Cells transformed by sarcoma viruses generally produce both leukemia and sarcoma virus particles, though some do not produce any virus. Particles with properties similar to those of RNA tumor viruses have been detected recently in human milk (*4*) and in cultured human rhabdomyosarcoma cells (*5*). Whether these viruses infect humans or have a role in human cancer is not yet known.

Mechanism of Cell Transformation by DNA Tumor Viruses

Extensive studies during the past 7 years have greatly enhanced our understanding of the mechanism of cell transformation by DNA tumor viruses. We describe the results of our study with the adenovirus system as a model. Of the 31 human adenoviruses, 12 can transform cells in culture, induce tumors, or both. Transformed cells, while not producing virus, do synthesize virus-specific tumor antigens (*6*). Similar antigens are also present early during productive infection (*1*, *6*). This observation first indicated that the viral genome may actively persist in the transformed cell.

The transformation of cells by a DNA tumor virus involves the integration of viral DNA sequences into the chromosomal DNA (text-fig. 1). For example, multiple copies of viral DNA sequences have been detected in cells transformed by various adenoviruses (*7*). These viral genes most likely function in transformed cells, for a large proportion (2–5%) of the messenger RNA (mRNA) in cytoplasmic polyribosomes is virus-specific (*8*). The adenovirus RNA sequences in transformed cells represent a restricted portion of the integrated viral genome (some of the "early" viral genes); they are transcribed together with contiguous cellular DNA as large polycistronic RNA molecules containing both cellular and viral RNA sequences (*9*).

Major unsolved problems are the identification of the virus-coded proteins in cells transformed by DNA tumor viruses and the understanding of how these proteins induce and maintain the transformed properties of the cell. Although these proteins from transformed cells may be isolated, identifying and controlling their functions in cell transformation will almost certainly be more difficult until we supplement our meager knowledge of the synthesis and regulation of macromolecules in mammalian cells.


NORMAL CELL
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Isolation of labeled mRNA
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?
viral polyribosomes
viral-coded proteins
isolated viral mRNA*
* labeled with isotope

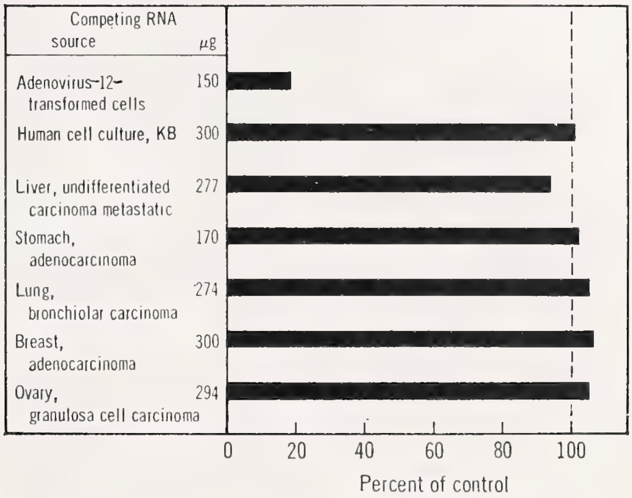
TEXT-FIGURE 1.—Mechanism of cell transformation by DNA tumor viruses and analysis for intracellular virus-specific RNA.

Detection of DNA Tumor-Virus Genetic Information in Human Cancers by Molecular Hybridization

Virus-specific RNA in cells transformed by DNA tumor viruses can be detected by DNA-RNA hybridization (text-fig. 1). The specificity of the annealing reaction is so precise that hybridization affords the opportunity to detect viral RNA sequences in human cancers. We have found that the direct hybridization of labeled human cancer RNA with viral DNA is not adequate for the following reasons (*10*): 1) Human cancer cells derived from tissues removed in biopsy or autopsy generally incorporate insufficient amounts of ^{3}H-uridine or ^{32}P-inorganic phosphate into their RNA molecules; and 2) the labeling of human cancer cells established in culture is not practical, since only 5% permanent cultures have been established in our laboratory and it is often difficult to be certain that the cultured cell is indeed the cancer cell.

A second approach requires the use of RNA polymerase of *Escherichia coli* to synthesize highly radioactive, virus-specific RNA with the use of a viral DNA template. The complementary viral RNA can then be annealed with DNA extracted from human cells. This method has been used to detect Epstein-Barr viral DNA in Burkitt's lymphoma tissues (*11*, *12*). However, it has limitations, for the high background hybridization of complementary viral RNA with normal cellular DNA makes it difficult to detect a few gene copies per cell.

A third method, molecular hybridization-competition analysis (table 2), is especially useful for screening many human tumors. It provides a general approach for testing the 100 known DNA viruses as etiological agents of cancer or other diseases. It is based on the premise that viral RNA sequences, present in RNA specimens from human cancers, will block the annealing of radioactive virus-specific RNA to viral DNA. This assumption has been verified with the use of RNA from tumors induced in newborn hamsters by the human adenoviruses. In 1967, we first applied this procedure to the analysis of human cancers. Over 200 RNA specimens from human cancers, from 19 different organs, were competed with radioactive RNA from cells transformed by representatives of 3 different groups of oncogenic human adenoviruses. We obtained no evidence for virus-specific RNA in these cancers. For example, as shown in text-figure 2, homologous, transformed-cell, viral RNA competed effectively; hybridization of radioactive viral RNA was reduced to 18% of control values. In contrast, RNA from cancer of the liver, stomach, lung, breast, and ovary did not compete with viral ^{3}H-RNA from transformed cells (90–105% of control). While these results suggest that adenoviruses do not cause human cancer, they do not eliminate this possibility. Although adenovirus-transformed cells in culture contain about 10,000 viral RNA molecules and the tests of human cancers may be sensitive enough to detect 1,000 viral molecules, fewer viral RNA molecules might occur in human cancers. More sensitive hybridization tests are in progress with radioactive viral RNA synthesized *in vitro* with the *E. coli* RNA polymerase.



TEXT-FIGURE 2.—Results of molecular hybridization-competition analyses for human adenovirus-12 genetic information in human tumors (*10*). RNA from human tumors was analyzed for its ability to prevent the hybridization of radioactive RNA from adenovirus-12-transformed cells with adenovirus-12 DNA.

Mechanism of Cell Transformation by RNA Tumor Viruses

Until the summer of 1970, very little information was available concerning the mechanism of cell transformation by RNA tumor viruses. Indirect experiments with inhibitors had implicated DNA synthesis as a required step in cell transformation (*1*). The exciting discovery of RNA-dependent DNA polymerase in RNA tumor-virus particles explained these observations (*14*, *15*). It now seems likely that a DNA copy of the viral genome is formed after infection and that such DNA is integrated into cellular chromosomes.

Using the ^{3}H-DNA product of the reverse transcriptase as a reagent in hybridization reactions, we have begun to study the synthesis and role of intracellular viral RNA, both in cell transformation and virus replication. It had been believed that

TABLE 2.—Hybridization-inhibition test for DNA virus genetic information in human tumors*

Step 1

$$\text{Viral DNA} + \text{human tumor RNA} \xrightarrow[\text{20 hours}]{66^\circ\text{C}} \text{DNA-RNA hybrid}$$

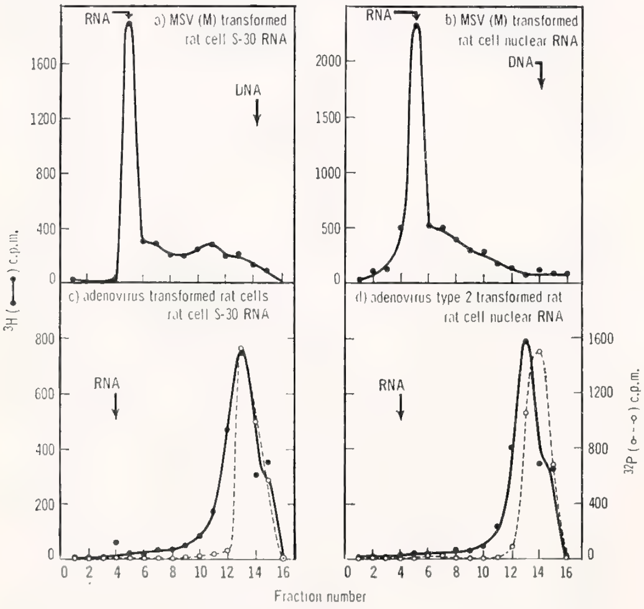
Step 2

$$+ \text{ virus-specific RNA}\dagger \xrightarrow[\text{20 hours}]{66^\circ\text{C}} \text{DNA-RNA hybrid}\dagger$$

*From Green (*13*).

†Radioactive molecules. Positive reaction = inhibition of radioactive hybrid formation; negative reaction = no inhibition of radioactive hybrid formation.

cells transformed by RNA tumor viruses possessed little, if any, virus-specific RNA, for many investigators failed to detect the characteristic 70S virion RNA within the cell. By hybridization analysis, this laboratory first demonstrated that both virus-producing and nonproducing cells transformed by the murine sarcoma virus (MSV) synthesized large quantities of virus-specific RNA (*16*). As shown in text-figure 3, hybrids formed between the ^{3}H-DNA product and cellular virus-specific RNA species banded at the position of RNA in cesium sulfate density gradients, for they consisted of RNA hybridized to much smaller DNA molecules. Both cytoplasmic and nuclear RNAs from MSV-transformed rat cells contained MSV-specific RNA; control adenovirus-transformed cells did not contain MSV-specific RNA sequences. Moreover, virus-specific RNA was detected in cytoplasmic polyribosomes, which suggests that such RNA serves as a message for the synthesis of virus-specific proteins (*17*).



TEXT-FIGURE 3.—Detection of virus-specific RNA in MSV-transformed cells. RNA samples were hybridized with the MSV DNA product and then analyzed by equilibrium centrifugation in cesium sulfate density gradients (*16*).

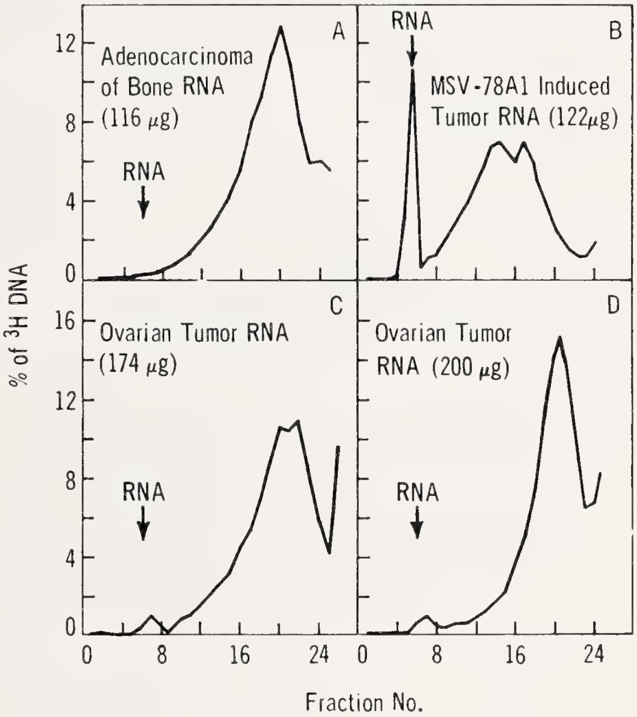
Thus the data available now point to great similarities in the induction and maintenance of the transformed state by RNA and DNA tumor viruses. Although the evidence is much more complete for DNA tumor viruses, in both instances sequences of the viral genome are apparently integrated into cellular DNA and a portion of the viral genome is transcribed to form virus-specific RNA molecules that function in the synthesis of virus-coded proteins. Some of these viral proteins most likely are important in inducing and maintaining the transformed properties of the cell.

Analysis of Human Cancers for RNA Tumor-Virus Genetic Information by Molecular Hybridization

The viral etiology of cancer in animals suggests that RNA tumor virus genetic information is involved in human cancer (*18*). The analysis of human cancers for human RNA tumor virus information requires both definitive identification and large-scale propagation of human tumor viruses in culture. Such goals have not yet been achieved; therefore, current efforts are directed toward analysis of human tumors for genetic information specific for murine, feline, and primate RNA tumor viruses.

We proposed (*16*) that the radioactive DNA product of RNA tumor-virus particles can be used as a sensitive and specific reagent to test, by molecular hybridization, for the involvement of viral genetic information in human neoplasia and other diseases (table 3). The DNA product is annealed with purified RNA from human cancers, and 2 procedures are utilized to detect virus-specific RNA: hydroxylapatite chromatography and equilibrium centrifugation in cesium sulfate density gradients. We have used both methods, for each has distinct advantages as well as limitations (*2*, *16*). Hydroxylapatite chromatography uses differential elution by phosphate buffers to distinguish between double- and single-stranded nucleic acid molecules. With this sensitive method, the RNA from many tumors can be rapidly analyzed. In the second procedure, cesium sulfate centrifugation (text-fig. 3), the hybrid formed between high molecular weight RNA molecules and the 4-7S DNA product of the reverse transcriptase banded at a position close to that of free RNA. This method is laborious, since it involves 3 days of centrifugation and the collection and processing of many fractions; moreover, it requires both RNA and DNA of sufficient size to form sharp, well-resolved bands in the gradient. However, it has the advantage of detecting RNA sequences which are homologous to a small fraction of the sequences present in the DNA product.

Numerous studies utilizing these methods are in progress. We have tested the ability of RNA from human cancers to hybridize with the DNA products of the feline sarcoma virus (FeSV), feline leukemia virus (FeLV) grown in human cancer cells, the Mason-Pfizer monkey tumor virus (MPMV), MSV, and the RD-114 virus derived from the human rhabdomyosarcoma. Examples of positive hybridization with human ovarian cancers and the MSV DNA product are given in text-figure 4. RNA from the MSV-induced mouse tumor served as a positive control (10–30% hybridization), whereas RNA from an adenocarcinoma of the bone yielded negative results. The RNA from ovarian tumors annealed with 1–2% of the DNA product, as shown by the small peak at the density



Text-figure 4.—Detection of radioactive MSV DNA "hybrid" peaks in cesium sulfate density gradients after annealing the ^{3}H-MSV DNA product with RNA from 2 different ovarian human cancers, a human adenocarcinoma of the bone, and an MSV-induced mouse tumor.

Table 3.—Detection by molecular hybridization of RNA specific to RNA tumor viruses in human cancer

1) Preparation of viral ^{3}H-DNA copy of RNA genome:

$$^3\text{H-dTTP}^* + \text{dATP}^* + \text{dCTP}^* + \text{dGTP}^* \xrightarrow[\text{RNA-dependent DNA polymerase}]{\text{70S viral RNA, Mg}^{++}} \text{viral } ^3\text{H-DNA}$$

2) Hybridization of viral ^{3}H-DNA with human tumor RNA:

$$\text{Viral } ^3\text{H-DNA} + \text{human tumor RNA} \longrightarrow {}^3\text{H-DNA-RNA hybrid}$$

*dTTP is deoxythymidine triphosphate; dATP is deoxyadenosine triphosphate; dCTP is deoxycytidine triphosphate; and dGTP is deoxyguanosine triphosphate.

of RNA (text-figs. 4C, D) (*19*). Some RNAs from normal ovaries form distinct but smaller peaks. We also found similar evidence for the annealing of FeLV, RD-114, and MPMV DNA with human cancer RNAs (*19*). Spiegelman and colleagues recently detected similar peaks at the density of RNA, utilizing the DNA products of the Rauscher leukemia virus (*20*) and the mouse mammary tumor virus (*21*). These results suggest that a small proportion of the base sequences represented in the DNA product of some RNA tumor viruses is transcribed in human cancers. The significance of these findings and future directions for research in this area are discussed below.

Perspectives

We have described methods, utilizing molecular hybridization, for the detection of sequences specific for RNA and DNA tumor viruses in human cancers. Thus far, analyses of human cancers have not detected gene sequences of the human adenoviruses; however, the molecular hybridization technique must be further refined to exclude decisively the involvement of a small amount of virus-specific sequences. Since herpesvirus type 2 transforms cells in culture (*22*) and may be involved in the etiology of cervical cancers (*1*), these analyses must include the herpesvirus group.

The positive results obtained by the hybridization of RNA specimens from human cancers with the DNA product of MSV (*19*) as well as similar data with the Rauscher leukemia virus and the mouse mammary tumor virus (*20*, *21*), MPMV, RD-114, and FeLV (*19*) are of great interest. Although they suggest that RNA tumor-virus genetic information is expressed in human cancers, exact interpretation of these results is not yet certain. Four explanations are possible—explanations *a*, *b*, and *c* are trivial, whereas explanation *d* may lead to a greater understanding of cancer. *a*) The presumed hybrid may not be a true hybrid but may consist of poorly matched, partially homologous sequences between human cancer RNA and the DNA product. This possibility can be readily tested by physical studies on the hybrid, such as determination of its thermal melting profile. *b*) Recent studies (*23*, *24*) have shown that the 70S RNA of RNA tumor viruses contains adenylic-acid sequences of 100–200 nucleotides—poly (A). The DNA product may contain poly (dT) sequences, and the presumed hybrid may be a complex between cellular poly (A) and viral poly (dT). *c*) Since RNA tumor virus virions contain cell nucleic acids, the DNA product most likely contains both cell-specific and virus-specific sequences. Thus the presumed hybrid may be formed between cancer cell RNA and cell DNA sequences. *d*) The presumed hybrid may be a true hybrid between virus-specific DNA sequences and complementary RNA sequences that are enriched in certain categories of human cancers. These cellular RNA sequences may have originated from either viral genes or cellular genes homologous to viral sequences. The first possibility implicates viruses in human cancer, though this information is not sufficient to differentiate between a function as a causative agent or as a passenger virus. The second possibility is that suggested by the oncogene hypothesis; *i.e.*, specific gene sequences common to viruses and human cells are involved in cancer (*18*). This hypothesis is supported by the recent induction of endogenous RNA tumor virus information in rodent cells (*25–27*). Further studies will determine if certain RNA sequences are expressed specifically in neoplasia, as suggested by these nucleic-acid hybridization tests. The further characterization of these RNA sequences, the identification of their cellular functions, and the analysis of the regulation of these functions should lead to advances in understanding and controlling cancer.

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ADDENDUM[1]

During the past 6 years, we have seen 1) a tremendous increase in our understanding of DNA tumor viruses (adenoviruses, papovaviruses, herpesviruses) and RNA tumor viruses (retroviruses), and 2) the revolutionary development of technologies that can detect less than a single viral gene/cell in cancer tissue. The 31 human adenoviruses (Ad) form 5 DNA homology groups; 30 are tumorigenic and/or transform cells. Two new ubiquitous human papovaviruses, BKV and JCV, are tumorigenic in animals. There are at least 3 different human papillomaviruses, including HPV-1 (plantar warts), HPV-2 (hand warts), and EV (epidermodysplasia verruciformis lesions). Ubiquitous human herpesviruses, herpes simplex types 1 (HSV-1) and 2 (HSV-2), and cytomegalovirus (CMV) transform cultured cells.

Retroviruses can multiply through horizontal infection (exogenous) or are transmitted in the germ line (endogenous). Probably all higher organisms harbor endogenous retroviruses (not proved for humans).

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[1] William S. M. Wold is the co-author of this Addendum.

The general features of the organization of tumor virus genomes are known. The Ad "transforming genes" are located in the left approximate 7% of the viral genome. The simian virus 40 and polyoma virus-transforming genes are also in the "early" region. The transforming genes (*src*) of avian sarcoma viruses are located near the 3′ terminus of the viral genome.

Data have accumulated on virus-induced animal cancers. Herpesviruses cause a type of lymphoma (Marek's disease) in chickens and Lucké carcinoma in the frog. Papillomaviruses probably cause skin cancer in rabbits and alimentary and bladder cancers in cattle. As yet, no evidence has been shown that Ad or polyoma-type papovaviruses cause cancer in their natural host.

Considerably more cases of retrovirus-induced natural animal cancers are known. Feline sarcoma–leukemia virus complex (FeSV–FLV) and bovine leukemia virus (BLV) cause leukemia in outbred cats and cattle, respectively. Avian retroviruses cause sarcomas and leukosis. The exogenous gibbon ape leukemia virus (GALV) causes leukemia in gibbons. Endogenous viruses cause cancer in inbred murine species.

Despite our increased understanding of tumor viruses, it remains uncertain whether they play a causal role in human cancer. A role for the Epstein-Barr virus (EBV) in Burkitt's lymphoma and nasopharyngeal carcinoma is strongly suggested by 1) seroepidemiologic data, 2) EBV sequences in tumors, 3) the induction of lymphoma in marmosets, and 4) in vitro transformation of lymphocytes. Less convincing evidence links HSV-2 and perhaps anogenital papillomavirus with cervical carcinoma and hepatitis-B virus with liver cancer.

Evidence for human retroviruses and for the expression of retrovirus genes in human tumors is controversial. Scientists in several laboratories isolated retroviruses, indistinguishable from the baboon endogenous virus (BEV) and woolly monkey sarcoma virus (WoSV), from embryonic and malignant human cells; do they represent contaminants or human viruses?

Nucleic acid sequences partially related to Rauscher murine leukemia virus (R-MuLV) have been detected in human tumors, those related to R-MuLV, FeLV, WoSV, and BEV in human leukemias, and those related to the mouse mammary tumor virus (MuMTV) and the Mason Pfizer monkey virus (MPMV) were seen in human breast cancer. Low levels of particles with reverse transcriptase activity (not infectious) were detected in various human neoplastic tissues. Finally, reverse transcriptase was purified from a leukemic spleen and from breast cancer; the breast cancer enzyme is antigenically related to the MPMV reverse transcriptase. Several investigators have detected antibody in most normal sera that reacts with proteins of the GALV–WoSV and BEV groups; this finding suggested that a primate-related retrovirus commonly infects humans. However, others did not confirm these findings.

A most exciting and recent achievement occurred when three new genetically transmitted primate viruses, differing from BEV, were discovered by long-term co-cultivation of primate cells with human, bat, feline, or canine cells. These include the squirrel monkey and owl monkey viruses, a type D and type C retrovirus, respectively, of New World monkeys, and the langur retrovirus (a type D retrovirus of Old World monkeys). The belief that humans also harbor endogenous retroviruses has thus been reinforced.

Many studies established that virus-induced tumor cells in animals and cell culture retain viral transforming DNA sequences. Therefore, we studied the possible viral etiology of human cancer by molecular hybridization analysis of human cancer DNA or RNA for viral sequences. In 1972, the lower limit of sensitivity was about 1,000 viral RNA molecules/tumor cell. This was insufficient to detect a single transforming gene/cell; therefore "negative" data could not rule out a viral etiology. Since that time, remarkable improvements have been made in methodology; viral DNA's, labeled in vitro to high specific activities ($>10^8$ cpm/μg), are used to probe tumor DNA or RNA for viral sequences by liquid phase "saturation–hybridization." Papovavirus DNA probes ($3–5 \times 10^6$ daltons) can detect one copy of 5–10% (<1 gene) of the viral genome/tumor cell; thus negative tumors are almost certainly not virus induced. Ad transforming fragment probes (i.e., restriction endonuclease DNA fragments that harbor the virus-transforming genes) can detect one copy per cell of 0.5% (<1 gene) of the viral genome. Herpesvirus DNA probes ($\sim 100 \times 10^6$ daltons) can detect about one copy of 10% of the genome, but since transforming genes could represent 1% of the genome, negative data are inconclusive.

We have analyzed 150–200 human tumor DNA's by using each of the following radiolabeled Ad probes: group A *Eco*RI-C transforming fragment (left 16% of genome), group C *Hin*dIII-G transforming fragment (left 7.5%), and group B and group E (Ad4) Ad whole genomes. No viral sequences were detected. We also assayed DNA's from 166 human cancers and 7 malignant human cell lines for BKV DNA, and 150 cancer DNA's for DNA's of the plantar and common human papillomaviruses. No viral sequences were detected. Thus no evidence is available that Ad's or papovaviruses cause human cancer. However, these tumors represent only 50% of the cancer types found in the United States, so that much work remains with less common cancers and with other viruses to exclude human DNA tumor viruses as agents of human cancer. Our studies represent the first definitive data on the relationship of oncogenic human DNA viruses to human cancer.

Recently, we assayed 150 cancer DNA's for sequences of JCV, HSV-2, and CMV. With each virus type, some DNA's contained viral sequences. Although the significance of these preliminary studies is unknown, they are clearly of potential importance.

Our laboratory has recently adopted the sensitive and elegant Southern transfer procedure to assay human tumor DNA's for virus sequences (*1*). Briefly, tumor DNA restriction fragments, resolved by electrophoresis and transferred onto nitrocellulose paper, are annealed with [^{32}P]-labeled viral probe DNA and exposed to X-ray film. Virus sequences appear as dark distinct bands. This method requires only 10 μg of human DNA and can detect one copy of viral DNA of $0.2–1 \times 10^6$ daltons. Thus we can detect less than or equal to 1% per cell of the Ad or herpesvirus genomes.

With our increased understanding of tumor viruses, these major advances in technology to detect minute quantites of virus sequences in human tumors should lead in the near future to a final answer to whether viral sequences are associated with specific human cancers. The detection of viral sequences will not prove a virus etiology, but it will provide a strong rationale for further etiologic and possibly prophylactic studies.

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RNA Tumor Viruses—Terminology and Ultrastructural Aspects of Virion Morphology and Replication

CONFUSION continues to surround the terminology used to categorize members of the RNA tumor virus group. This is in part due to the fact that, when Bernhard looked at different kinds of mouse tumors with the electron microscope, he found 4 different morphologic types of particles associated with them (*1*). The 2 types related to mammary tumors he called A and B. The one associated with leukemia he called type C, and the one related to salivary gland tumors is called type D. The type D particle develops within the nucleus and represents a small DNA virus and will not be considered further. The type A particle was intracellular and consisted of 2 concentric spherical electron-dense shells with an electron-lucent center. (The shells are comparable to 2 adjacent and concentric layers of an onion.) These particles thus had a doughnut form in thin section. Their average diameter is 70 nm. The type B particle was extracellular and had a loosely fitting trilaminar envelope and an eccentric, electron-dense nucleoid. They average 110 nm in diameter. The type C particle was also extracellular with a loosely fitting envelope but possessed a central nucleoid. Type C particles approximate 100 nm in diameter.

These 4 particles were identified by letters of the alphabet presumably because, except for their morphology, little was known about them. Now we know that type B particles induce mammary tumors in mice so that we can think of them as virions representing a mammary tumor virus (MTV). Similarly, type C particles are virions representing a murine leukemia virus (MLV). Type C virions induce neoplasms of the avian leukosis-sarcoma complex and the feline and murine leukemia-sarcoma complexes. Virions representing polyoma virus and the K virus of mice are morphologically identical and are small DNA viruses (type D).

Since Bernhard's early observations, 2 types of A particles have been distinguished. Both have an average diameter of 70 nm. The first type is present in the cytoplasmic matrix of cells of mice carrying the MTV. They sometimes become the nucleoid of budding type B particles and are presently called intracytoplasmic A particles (*2*) (fig. 1). The second type bud into the cisternae of the endoplasmic reticulum. They are called intracisternal A particles and, while containing RNA, have not been demonstrated to possess biological activity (*2*) (fig. 2).

Most details of the formation of type B and type C particles are now well known, but again the terminology is confusing. As indicated above, type

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Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

B particles sometimes acquire intracytoplasmic A particles as nucleoids. More often the nucleoid is formed under the plasma membrane of a developing bud, first as a double-layered crescent or horseshoe profile (fig. 3) and finally as a double-shelled sphere (fig. 4). When the particle separates from the cell, it contains a central nucleoid with an electron-lucent center surrounded by 2 moderately electron-dense shells. Radiating spokes extend from the surface of the outer shell to the inner border of the envelope which is a unit membrane covered with spikes (fig. 5). Later changes result in a more even distribution of electron-dense material throughout the nucleoid and a shift of the nucleoid to an eccentric position (fig. 6).

Type C particles form in a manner similar to that described for type B particles. However, 2 differences may be noted: 1) Under the same conditions of preparation for electron microscopic observation the inner of the 2 layers forming the outer part of the nucleoid is much more electron dense than the outer layer (fig. 7). This makes it possible to distinguish developing type B particles from developing type C particles (*cf.* figs. 5, 8). In the mouse and cat, the newly released type C particle continues to have an electron-lucent center (fig. 8). 2) The "mature" type C particle possesses a more or less evenly electron-dense nucleoid in a central rather than an eccentric position (fig. 9). Surface projections are present on the type C particles of avian myeloblastosis virus and are sometimes seen on other type C particles, depending on the methods of preparation.

So much for the details of ultrastructure of virions and virion replication. The problem of terminology is something else. One view is that only extracellular virions with an electron-dense, eccentric nucleoid should be called type B particles, and only extracellular virions with an electron-dense central nucleoid should be called type C particles. Budding particles and extracellular particles with a nucleoid containing an electron-lucent center should be called enveloped A particles (*3*). The other view is that, from the time the developing type B and type C particles can be distinguished from one another and before they acquire electron-dense nucleoids, they should be called budding or "immature" type B and type C particles, "immature" being used without any implication as to infectivity. This position is based in part on the fact that, in the formation of most type B particles and all type C particles, no true intracellular type A particle is ever involved. The presence of a nucleoid with an electron-lucent center is not synonymous with the phrase "type A particle" (*4*).

To repeat—in one view, budding or newly released particles, whether destined to become either B or C particles, are called enveloped A particles because the nucleoid has a double shell and an electron-lucent center. In the other view, newly released particles destined to become B particles are called "immature" B particles. Newly released particles destined to become type C particles are called "immature" C particles.

In recent studies involving the details of type C particle formation, an interesting observation was made: Under conditions of constant but special fixation, and in the instances so far studied, type C virions indigenous in different species can be distinguished from one another. [The special fixation used was either chrome osmium (*5*) followed by 10% neutral formalin containing 0.5% uranyl acetate or 3% glutaraldehyde in Sorensen's buffer followed by chrome osmium. The results are not the same when osmic acid in phosphate buffer is used.] The basis for this distinction is in the thickness and relationship of the outer component (shell) of the developing nucleoid at the bud stage. This outer component is sometimes called the intermediate layer and is considered to consist primarily of viral protein. Murine leukemia virions, whether budding from mouse or human cells, show the same features of the intermediate layer: This layer is relatively thick (approximately 100 A) and separate from the more electron-dense, inner component of the nucleoid (fig. 10). Feline type C virions budding from either cat or human cells also possess a distinct intermediate layer but it is much thinner than that of the mouse virions (approximately 50 A) (fig. 11). Type C virions budding from cells of the ESP-I human tissue culture cell line are different from either the mouse or cat virions in that there is no clear distinction between the intermediate layer and the inner component of the nucleoid when either chrome osmium followed by 10% neutral formalin or 3% glutaraldehyde in Sorensen's buffer followed by chrome osmium is used as a fixative (fig. 12). When ESP-I virions are fixed in glutaraldehyde followed by osmic acid in phosphate buffer, the intermediate layer is clearly distinguished (fig. 13). On the other hand, type C particles budding from human cells of the McAllister RD-114 tissue culture cell line fixed in chrome osmium resemble cat virions more than they do the other two in that they possess a thin but distinct intermediate layer (fig. 14). These results indicate that, in the budding type C virions examined, the morphology and relationships of the intermediate layer component of the nucleoid are under viral-genome, not host-cell control. Finally, while it may seem obvious to some, it should be pointed out that the type or kind of fixation used in these and other ultrastructural studies has a direct bearing on the results obtained.

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ADDENDUM

Since the publication of the Editorial on RNA tumor viruses in 1972, discussions on terminology and classification have taken place (*1, 2*). These discussions resulted in the adoption of the name "retroviridae" by the International Committee on the Nomenclature of Viruses (ICNV) to characterize those viruses containing reverse transcriptase. Also articles describing type C particles in the placentas of various primates from marmosets to humans have been published (*3–8*). These particles differ in ultrastructural detail from typical type C particles in that the electron lucent space usually present between the viral envelope and the nucleocapsid is not clearly identifiable in them. Also the intermediate layer, so clearly evident at some stage of development or maturation in type B and C particles, cannot be visualized in the placental particles (*9*).

One of the problems facing investigators attempting to characterize these particles further has been their relative scarcity. Until recently, no long-term cell lines reproducing these particles had been described. However, Bronson et al. (*10*) described a cell line derived from a metastasized human testicular tumor that produced particles possessing ultrastructural characteristics identical to those of the placental particles. Although few particles are produced by this cell line, too few for immunologic or biochemical characterization, the results surely suggest that further studies involving the development of additional choriocarcinoma cell lines of either placental or testicular tumor origin are in order.

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All figures except 10, 12, and 13 are from material fixed in chrome osmium followed by 10% neutral formalin containing 0.5% uranyl acetate. Figures 1 and 2 are approximately × 100,000. All other figures are approximately × 140,000.

Figure 1.—Group of intracytoplasmic A particles in mammary tumor cell of BALB/c mouse foster-nursed on C3H/An mouse.

Figure 2.—Intracisternal A particles in cell of plasma cell tumor of BALB/c mouse. Budding particle is at *upper right*.

Figure 3.—Type B particle budding from mammary tumor cell of BALB/c mouse carrying MTV. *Note* that inner component of developing nucleoid is only slightly more electron dense than outer component or intermediate layer.

Figure 4.—Late bud of type B (enveloped A) particle from BALB/c mouse carrying MTV. *Note* similar electron densities of the 2 components of nucleoid.

Figure 5.—Free "immature" type B (enveloped A) particle with spikes on surface, electron-lucent center in the nucleoid, and spokes radiating from outer surface of nucleoid to inner surface of envelope.

Figure 6.—Two type B particles with eccentric nucleoids.

Figure 7.—Type C particle budding from surface of cell in bone marrow of BALB/c mouse that developed leukemia after injection of Moloney strain leukemia virus. *Note* high electron density of inner component of nucleoid. *Cf.* with figures 3, 4, 5.

Figure 8.—Two free "immature" type C (enveloped A) particles in space between platelet-demarcating membranes of megakaryocyte from same material from which figure 7 was taken. *Note* high electron density of inner component of nucleoids and distinctness of thick (100 A) outer component.

Figure 9.—Type C particle in extracellular space. Nucleoid is central.

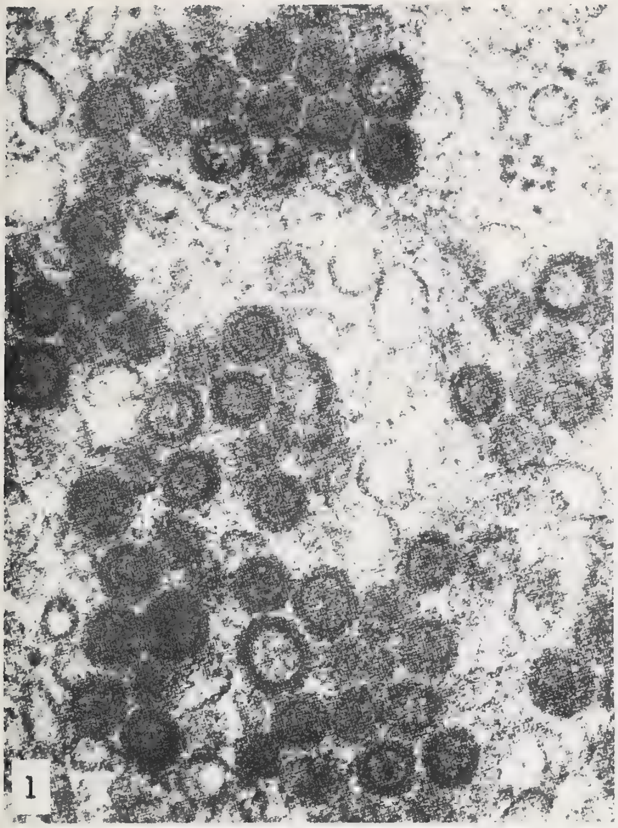
Figure 10.—Late bud of type C particle in tissue culture of human embryo kidney cells infected with the strain of Rauscher leukemia virus that grows in human cells. *Note* distinctness of outer component of nucleoid (intermediate layer). This is similar to its appearance in figures 7, 8 but different from figure 12. Tissue fixed in 3% glutaraldehyde in Sorensen's buffer followed by chrome osmium.

Figure 11.—Type C particle budding from human embryo lung cell in tissue culture infected with feline leukemia virus. *Note* thin (approximately 50 A) but distinct outer component of nucleoid [from fig. 9 in (*6*)].

Figure 12.—Type C particle budding from cell of tissue culture line ESP-I. *Note* no clear distinction between the 2 components of nucleoid. Tissue fixed as in figure 10.

Figure 13.—Type C particle lying free in intercellular space in culture of ESP-I. *Note* clear separation between the 2 components of nucleoid. Fixed in 3% glutaraldehyde followed by 1% osmic acid in phophate buffer.

Figure 14.—Type C particle budding from cell of tissue culture line RD-114. *Note* thin but distinct outer component of nucleoid. *Cf.* with figures 7, 8, 10, and 11.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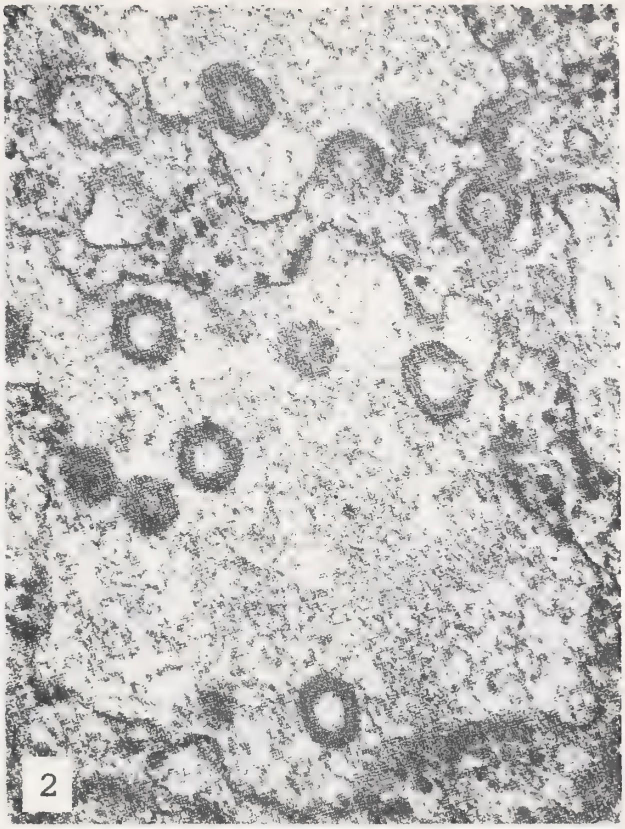
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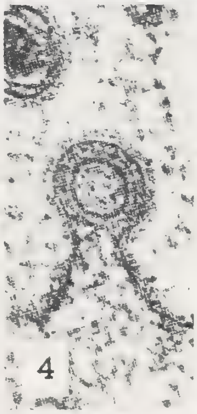
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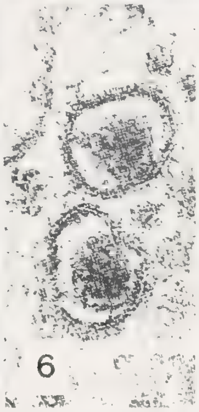
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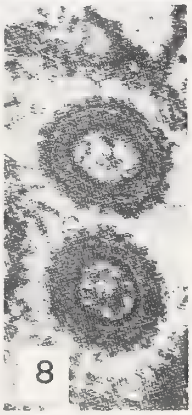
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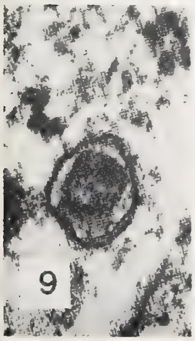
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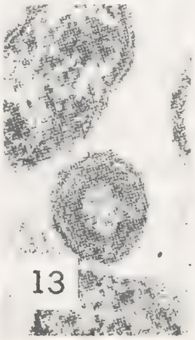
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GUEST EDITORIAL——by **Tadao Aoki, *M.D., Ph.D*** [1, 2]

JNCI 52:1029–1034, 1974

Murine Type-C RNA Viruses: A Proposed Reclassification, Other Possible Pathogenicities, and a New Immunologic Function[3]

SYSTEMATIC STUDIES of both avian and murine type-C RNA viruses were initiated by the discovery of the avian type-C RNA virus, Rous sarcoma virus (RSV), in 1911 (*1*). The characteristics of type-C viruses have been investigated extensively by comparison of the avian and murine systems: e.g., *a*) the host range of viruses, C/O, C/A, C/B, C/C, C/D, C/E, and C/F in the avian system (*2–4*), and B-, N-, and NB-tropic in the murine system (*5*); *b*) the intraviral group-specific (gs) antigens (*6–8*); *c*) the specific intraviral enzyme, RNA-dependent DNA polymerase or reverse transcriptase (*9*, *10*); and *d*) the interaction of sarcoma viruses and helper viruses, resulting in modification of sarcoma virus host range by the acquisition of viral envelopes from different species or helper viruses (*11*). In this editorial, I shall mainly review the murine system.

Intraviral gs antigens and viral reverse transcriptases have provided the most significant markers to date for detection and specification of type-C viruses. On the other hand, for the prevention or prophylaxis against virus infection and/or oncogenesis, virus envelope antigens (VEAs) must be utilized to produce host immunity against type-C viruses. Here, I would like to deal mainly with VEAs, since recent methods of analysis by immunoelectron microscopy have made it feasible to elucidate these antigens in detail (*12–14*). The broad classification of murine type-C virus-associated antigens on the surface of viruses and their infected cells into 2 major groups—Gross (G) and Friend, Moloney, Rauscher (FMR)—has long been accepted as a classic concept (*15*). But solid knowledge of the antigenic relationships, not only among G and FMR but also among other type-C viruses, still is lacking. Most murine type-C viruses have been considered leukemogenic. Since the discovery of murine sarcoma virus (MSV) (*16*), however, it has been expected that these viruses may induce other types of tumors.

Additionally, a cell-surface alloantigen or "differentiation antigen" PC1[4] on the surface of malignant as well as normal cells was originally considered to be determined by the cellular genome, because of its antigenic specificity and its mouse tissue and strain distributions (*18*). Recent immunologic and virologic studies of type-C viruses have been altering this concept (*19*). A newly detected extracellular type-C virus associated with murine plasmacytomas has been found to induce PC1 antigen (*see* below), which suggests a new function of type-C viruses.

Perhaps now is the appropriate time to reexamine and reconsider some previous concepts concerning type-C viruses. The following subjects are of particular interest: 1) The diversity of type-C VEAs, including the relationship between surface antigens of cells and viruses; 2) possible pathogenic roles of type-C viruses other than in leukemias and sarcomas; and 3) the induction of alloantigens or differentiation antigens on the cell surface by type-C viruses.

TYPE-C VIRUS-ASSOCIATED SURFACE ANTIGENS

The classification of murine leukemia virus (MuLV)-associated cell-surface antigens into 2 major categories, G and FMR, was first established by criteria of humoral cytotoxicity (*20*, *21*) and indirect immunofluorescence (*22*, *23*); a somewhat similar classification of MuLV envelope antigens was suggested by neutralization tests (*24*, *25*). However, the unexpectedly broad spread of G-MuLV in almost all strains of mice (*26*) has indicated that classification

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[4] The term "differentiation antigen" refers to an antigen that appears only on certain types of cells, and whose expression thus appears to be conditional on a particular pathway of differentiation (*17*). The differentiation antigen PC1 is present on plasmacytomas, on normal plasma cells, and on cells of the liver, kidney, brain, and lymph nodes (*18*). To demonstrate this antigen, cytotoxic antibody was produced by immunization of (C57BL/6 × DBA/2)F_1 mice with BALB/c mineral oil-induced plasmacytoma MOPC-70A. Mouse strains can be classified into 2 groups—PC^+ and PC^-—according to the presence or absence of the antigen on normal cells.

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of MuLV should be amplified or modified. FMR leukemia cells can completely absorb cytotoxic activity from FMR antiserum and only weakly from G antiserum, in contrast to the fact that G leukemia cells can absorb G antibody but not FMR antibody (*15*). This indicates the following 2 possibilities: 1) the FMR system is contaminated with G-MuLV and/or 2) besides their own individual specific antigens, the FMR system shares a common antigen with the G system but not vice versa. As immunologic techniques are developed and refined, more detailed information on type-C VEAs has emerged, suggesting a more appropriate reclassification of these antigens. In particular, the recent improvement of immunoelectron microscopy has facilitated the demonstration of the diversity as well as the partial uniformity of type-C VEAs and virus-associated cell-surface antigens.

VEAs

Very recently, the specificity of VEAs was studied by both neutralization tests (*27*, *28*) and immunoelectron microscopy (*29*). The findings may be summarized as follows (table 1): *a*) gsVEA is the broadly common VEA recognizable by immunoelectron microscopy with the serum from selected aged autoimmune NZB mice (*14*, *19*) and by neutralization tests with the rat or rabbit anti-MuLV serum (*30*); *b*) subgroup-specific VEAs (sub-gs VEAs), less broad VEAs are common among some groups of type-C viruses, as categorized by Gomard et al. using mouse and rat antisera (*28*) and by us using rat antiserum preabsorbed in vivo in normal mice to remove natural xenoantibodies to normal mouse components (*19*); *c*) type-specific VEAs (tsVEAs) are individually specific among a variety of type-C viruses, even within the FMR group—Eckner and Steeves (*27*) described that M-MuLV pseudotypes of helper-dependent Friend spleen focus-forming virus [SFFV(MolLV)], as rescued by M-MuLV, were effectively neutralized by M antiserum but not by F-typing serum, although SFFV(MolLV) was coated with the M-MuLV envelope and contained the intraviral components of F-MuLV. Similar findings were obtained among G-, F-, M-, R-, and Graffi-MuLV. These results nicely correspond to our results, obtained by immunoelectron microscopy, with the use of mouse antisera (*19*). Moreover, mice can sometimes recognize other specific VEAs in addition to tsVEAs in the same group of type-C viruses, which indicates the presence of variant tsVEAs classified as subtype-specific VEAs (sub-tsVEAs). Some discrepancies among the proposed classifications may be due to the methods used for the detection of VEAs.

It must be emphasized that endogenous type-C viruses of BALB/c somatic and plasmacytoma cells consist of at least 2 different populations, xVEA$^+$ and x1-VEA$^+$, whereas exogenous B-, or NB-tropic type-C viruses originate from BALB/c leukemias, i.e., Rauscher leukemias, Moloney leukemias, and others carry individually specific VEAs. These type-C viruses released from and/or associated with various cell lines contain >1 virus population having individually specific VEAs (*29*). In other words, they harbor either a mixture of 2 different endogenous or exogenous type-C viruses, a mixture of endogenous and exogneous type-C viruses, or hybrid viruses.

Type-C Virus-Associated Cell-Surface Antigens

Besides the common specific antigen, individual specificities of cell-surface antigens among G, F, M, and R leukemias have been clarified to some extent in the mouse and rat systems. Cell-surface antigens of mouse G, F, M, and R leukemias were recently found by humoral cytotoxicity tests to possess individual specificities, though tumor-associated transplantation antigens (TATAs)[5] showed slight cross-reaction among these leukemias (Ting C-C, Herberman RB, personal communication). In the rat, however, slight cross-reaction among these leukemias was demonstrated by cytotoxicity tests (*31*), but individual specificities were found in TATAs (*32*). The discrepant results may be derived from the different methods and different species used in the 2 studies.

The individual specificities of VEAs in the mouse are substantiated by the facts that BALB/c radiation-induced leukemia BALB.RL♂1 cells release specific type-C viruses which may produce leukemia-specific X.1 cell-surface antigen (*33*), and that spontaneously transformed BALB/3T3 S2 clones, which are also spontaneously releasing specific type-C viruses (*34*), form PC1 cell-surface antigen (*13*). These 2 cell-surface antigens, X.1 and PC1, differ from known surface antigens on the typical MuLV-induced BALB/c leukemia cells.

Relationship Between VEAs and Virus-Associated Cell-Surface Antigens

Do VEAs of type-C viruses exist on the cell surface without showing any morphologic sign of virus budding? Type-C virus-producing cells may have cell-surface antigens common to VEAs besides VEAs themselves on the envelopes of budding and complete viruses. Actually, NZB serum reacts mainly with VEAs but not with the cell surface, as previously reported in the mouse G leukemia system (*35*). This serum was recently shown to react on rare occasions with minute areas on the surface of cells releasing type-C viruses (*36*), which suggests 3 probabilities: *a*) The NZB serum also reacts with some classes of G cell-surface antigens; *b*) type-C viruses initiate their synthesis beneath the plasma membrane before showing any morphologic sign of budding, indicating the production of VEA on the cell surface itself; or *c*) after budding, type-C viruses leave their VEA on the cell surface. Of these 3 probabilities, the second seems

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[5] In a restrictive sense, tumor-specific transplantation antigen (TSTA) implies tumor-specific antigens appearing on the cell surface after malignant transformation but does not include virus-associated or fetal antigens. Recently, the term "tumor-associated transplantation antigen" (TATA) often has been used in a broader sense which includes virus-associated or fetal antigens. To distinguish virus-associated transplantation antigens from TSTA, the term "virus-specified transplantation antigen" (VSTA) has been proposed by L. W. Law et al. (personal communication).

TABLE 1.—*New classification of antigens on the envelope of murine type-C (VEAs) demonstrated by immunoelectron microscopy, compared with classifications by neutralization tests*

Antiserum	New designation of antigens detected	Description		
		Aoki et al. (*19*) (immunoelectron microscopy)	Eckner and Steeves (*27*) (neutralization tests in vivo)	Gomard et al. (*28*) (neutralization tests in vivo)
Serum of autoimmune aged NZB mice.	gsVEA	A common VEA among all individual viruses		A common VEA among all MuLV.
Rat or rabbit anti-type-C viruses.*	sub-gsVEA	A common VEA of individual groups of MuLV† of plasmacytoma-associated viruses of other unknown viruses‡		Common VEAs in 3 subgroups of type-C viruses.
Mouse anti-type-C viruses	tsVEA	VEA carrying individual specificities	Individual specificities of VEAs between M-MuLV and F-MuLV.	Probable individually specific VEAs.
Mouse anti-type-C viruses	sub-tsVEA§	Further individually specified tsVEA		

*These antisera also recognize some tsVEA (*14*).

†MuLV identified on p. 5 including G-, F-, M-, and R-MuLV.

‡This group will be divided into more subgroups.

§One mouse typing serum occasionally reacts with VEAs of several different type-C viruses; e.g., the M-typing serum reacts with 100% M-MuLV and 44%xVEA$^+$ viruses [*see* (*19*)], indicating the necessity of further subdivision of tsVEA.

most likely, since a glycoprotein isolated from the surface of cultured nonproducer lymphocytes from an NZB mouse has been revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis to be antigenically common to that on the surface of Scripps leukemia virus (SLV), a type-C virus purified from the same cell line (*37*).

A possibly analogous circumstance has been definitely clarified in the avian sarcoma system (*38*); i.e., by immunoelectron microscopy, chicken antiserum against the Bryan strain of RSV (B-RSV) has been found to react positively, not only with the surface of B-RSV-infected transformed chick-embryo cells and of B-RSV (producers) but also with the surface of viable uninfected chick-embryo cells (nonproducers) having gs antigen and helper activity. In addition, the neutralizing activity of this antiserum to the envelope of B-RSV was absorbed with the nonproducer cells, indicating the presence of an antigen common to B-RSV envelope antigen on normal chick-embryo cells. The removal of antiserum activity by absorption was also confirmed by immunoelectron microscopy. In contrast, both virus-neutralization tests and immunoelectron microscopy showed that normal chick-embryo cells lacking gs antigen and helper activity did not react with the antiserum. Therefore, some VEAs of murine type-C viruses possibly are on the surface of mouse cells which carry the viral genome, phenotypically expressing at least intraviral gs antigen but neither budding nor complete viruses.

In any case, for the prevention of or prophylaxis against oncogenesis by type-C viruses, either a broadly common VEA or a mixture of individually specific VEAs must be utilized for active and/or passive immunization of hosts. Furthermore, common sub-gsVEAs may be utilized to detect type-C viruses belonging to host species, if there are such species-specific type-C viruses.

POSSIBLE PATHOGENICITY OF TYPE-C VIRUSES OTHER THAN INDUCTION OF LEUKEMIAS AND SARCOMAS

An etiologic role of type-C RNA viruses has been proved by the induction of brain tumors in mice, e.g., glioblastomas, astrocytomas, and their variants, by intracerebral injection with the Schmidt-Ruppin strain of RSV (*39*, *40*). This finding encourages us to investigate other pathogenicities of type-C viruses.

Administration of mineral oil to sensitive strains of mice easily induces plasmacytomas which usually release extracellular type-C viruses (*41*). A BALB/c plasmacytoma MOPC-70A produces many type-C viruses possessing a specific envelope antigen, xVEA (*42*). This antigen distinguishes these viruses from other murine type-C viruses. However, plasmacytomas developed in several PC$^+$ and PC$^-$ strains sensitive to mineral oil-plasmacytoma induction are occasionally accompanied by some as yet uncharacterized type-C viruses, in addition to xVEA$^+$ viruses (*41*). Either one or both of these viruses may be causatively related to plasmacytoma induction. This possibility is indirectly substantiated by the following observations: 1) Spontaneously transformed BALB/3T3 S2 clones release $\geqq 1$ virus populations either spontaneously or by the use of the thymidine analogues, bromodeoxyuridine (BrdU) and iododeoxyuridine (IdU) (*13*). One of these is identical with xVEA$^+$ virus (*41*), which suggests that most, if not all, BALB/c cells contain the viral genome in latent form. Regardless of the state of virus maturation, the viruses may contribute to the development of plasmacytomas and other neoplasms. 2) Intraperitoneal injection with mineral oil creates an abnormal tissue environment in the peritoneal cavity which permits the growth of plasmacytomas (*43*). Additionally, mineral oil appears to activate the viral genome to release complete endogenous type-C viruses from primary plasmacytomas (*41*), as BrdU or IdU does. Therefore, these findings lead to the hypothesis that the xVEA$^+$ type-C viruses may play either a primary role in the induction of plasmacytomas or at least a collaborative role in the mineral oil induction of plasmacytomas. To prove this hypothesis, isolates from plasmacytomas are being inoculated into sensitive mice to examine whether the mice will develop plasmacytomas. This study is still in progress. Thus, one can no longer regard the oncogenic role of type-C viruses as simple and monopathogenic but rather must consider it as complex and possibly associated with multiple normal and pathogenic states.

INDUCTION OF CELL-SURFACE DIFFERENTIATION ANTIGENS BY TYPE-C VIRUSES

Major histocompatibility antigens or differentiation antigens, such as H-2, θ, L_Y, TL, PC1, and others, have been considered to be conferred by the cellular genome, based on their specificities, tissue and strain distributions, and genetic behavior (*18*). Since a differentiation antigen PC1 on certain cell surfaces has been found to be induced by xVEA$^+$ type-C virus or by its genome, a reconsideration of the origin of some histocompatibility or differentiation antigens is demanded from a virologic standpoint. For example, PC1-inducing type-C viruses carry xVEA that differs from VEAs of other known typical MuLV and MSV. The reasons PC1 antigen is considered to be induced by the xVEA$^+$ virus or by its genome are described elsewhere (*19*). Briefly, 1) both PC$^+$ and PC$^-$ strains of mice naturally produce antibody against PC1 (*44*); 2) spontaneously transformed BALB/3T3 S2 clones release endogenous xVEA$^+$ and x1-VEA$^+$ viruses spontaneously or by the use of BrdU or IdU, and change from PC$^-$ to PC$^+$ (*13*); and 3) some mineral oil-induced primary plasmacytomas in PC$^-$ strains acquire PC1 antigen and release xVEA$^+$ type-C viruses, but some other plasmacytomas of PC$^-$ strains neither form PC1 antigen nor release xVEA$^+$ viruses (*41*).

PC1 antigen-positive cells therefore either release complete xVEA$^+$ type-C viruses or contain the viral genome in a latent form, which suggests that xVEA$^+$ virus or its genome exists in a form somewhat similar to the episome[6] in the *Escherichia coli* system (*45*). Like the fertility (F) factor of *E. coli*, xVEA$^+$ viruses are present in the integrated or the free form in the cells of PC$^+$ strains of mice. PC$^+$ mice seem to have a high frequency recombination (Hfr) system similar to that observed in *E. coli* (*46*, *47*). In the *E. coli* K12 system, $(F^+M^-B^- \times F^-T^-L^-B_1^-)$ F_1[7] hybrids form F^+ recombinants 1000 times more frequently than usual recombinants. Similarly, $(PC^+ \times PC^-)F_1$ mice are PC$^+$. The similarity of the 2 systems described above can be explained very nicely in the system of nonproducers of viruses; the xVEA$^+$ virus genome integrates so firmly that the phenotypic expression of PC1 shows Mendelian inheritance, but its allele has not yet been determined (*18*). Two considerable exceptions are that *a*) the episome was not transferred with *E. coli*-free agents, whereas PC1 antigen may be induced by infection with xVEA$^+$ viruses, and *b*) F^+ *E. coli* converts to F^-, whereas PC$^+$ cells have never been changed to PC$^-$ by any means (the TL system is different from the PC system concerning this point, *see* below). The 2 systems are compared schematically in text-figure 1.

In the thymus-leukemia (TL) system (*48*), a mechanism of TL antigen induction similar to that of PCl has long been postulated (*17*). TL antigen shows antigenic modulation; the phenotype of TL antigen on the leukemia cells is changed from TL$^+$ to TL$^-$ for as long as TL antibodies are present (*17*, *49*). This phenomenon is similar to the reversible conversion of episomes but contrasts with the PCl antigen system. However, despite extensive studies, the responsible type-C virus has not yet been detected, and efforts to establish the mechanism are continuing.

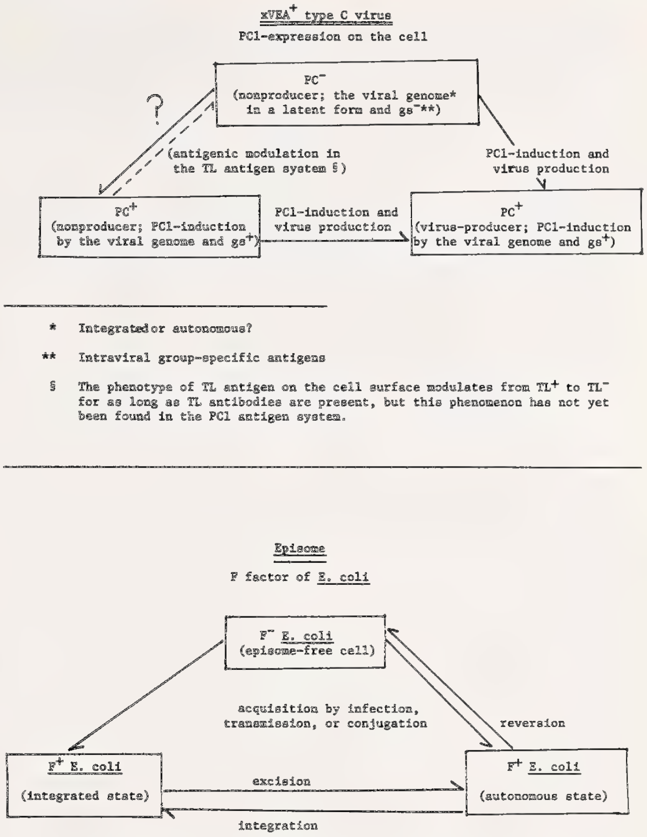
The probability that some alloantigens or differentiation antigens are induced by type-C viruses opens a way to reconsider the possible relationship of these antigens and related viruses. Perhaps the suggestive comparison with the function of the episome in the *E. coli* system may prove helpful in defining a new function of type-C RNA viruses.

COMMENTS

Visualization of antigenic sites on the surface of cells and viruses at the high resolution of immunoelectron microscopy has facilitated analyses of the immunologic and etiologic relationships between normal as well as malignant cells and type-C viruses. Although type-C viruses related to human tumors have not yet been discovered despite tremendous efforts, studies of type-C viruses in other species have provided and will continue to provide fundamental knowledge which ultimately may be applicable to the human system. Thus the reclassification of murine type-C viruses, proposed here on the basis of the unexpected diversity of their envelope antigens (*see* table 1), may prove of some general value in clarifying their virologic and immunologic functions.

ADDENDUM

While this paper has been in press, the host range of mammalian type-C viruses has been extended to include X- and S-tropisms.


xVEA+ type C virus
PC1-expression on the cell
PC−
(nonproducer; the viral genome* in a latent form and gs−**)
?
(antigenic modulation in the TL antigen system §)
PC1-induction and virus production
PC+
(nonproducer; PC1-induction by the viral genome and gs+)
PC1-induction and virus production
PC+
(virus-producer; PC1-induction by the viral genome and gs+)
Episome
F factor of E. coli
F− E. coli
(episome-free cell)
acquisition by infection, transmission, or conjugation
reversion
F+ E. coli
(integrated state)
excision
integration
F+ E. coli
(autonomous state)

* Integrated or autonomous?

** Intraviral group-specific antigens

§ The phenotype of TL antigen on the cell surface modulates from TL$^+$ to TL$^-$ for as long as TL antibodies are present, but this phenomenon has not yet been found in the PC1 antigen system.

TEXT-FIGURE 1.—Schemas comparing xVEA+ type-C virus in the PC1 antigen system and the episome in the *E. coli* system (*see* text).

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[6] Episomes are bacteriophages and genetic elements of bacteria which are defined by their ability to replicate in either the autonomous or the integrated state and to shift between these alternative states (*45*). The F factor of *E. coli* can be found either in the integrated state, replicating as part of the host chromosome, or replicating autonomously.

[7] F+ = F factor positive; M, B, T, L, and B_1 = methionine, biotin, threonine, leucine, and vitamin B_1 requiring, respectively.

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ADDENDUM

The rapid development of analytical techniques has revealed the complexity of antigens associated with type C RNA virus. Particularly by immunochemical analysis, some classes of type C RNA VEA have been identified and isolated on the basis of molecular weight and antigenic specificity [*see* (*1*, *2*)]. These antigens are protein or glycoprotein and correspond to the classification described in the Editorial, e.g., most MuLV-gp70 are gsVEA or subgsVEA and most MuLV p15 and p12 are tsVEA. Further detailed classification may be established in the future.

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GUEST EDITORIAL——**by Richard A. Steeves**[1]

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Spleen Focus-Forming Virus in Friend and Rauscher Leukemia Virus Preparations[2]

RECENT ADVANCES in the biochemistry (*1*) and immunology (*2–4*) of RNA tumor viruses have spurred a remarkable interest in these agents, and effective communications about them will require a thorough understanding of their biology and consistent use of a logical terminology (*5*). This is especially needed for the murine leukemia viruses (MuLV's) isolated by Friend (*6*) and Rauscher (*7*), usually called simply Friend and Rauscher leukemia viruses (F-MuLV and R-MuLV), respectively.

It is well established that Friend and Rauscher virus preparations are distinct from other MuLV's in the rapidity with which they induce hepatosplenomegaly and erythroleukemia in susceptible strains of mice infected as adults (*6–9*). Another property of Friend and Rauscher virus preparations is their capacity to induce macroscopic foci of primitive erythroid cells in the red pulp of the spleen (*10, 11*). This property, which is unique to F-MuLV and R-MuLV, could be due to an acquired genetic change in a homogeneous virus population or to the presence of another virus in a heterogeneous virus population. Recent evidence (*12–14*) supporting the latter alternative has shown that the spleen focus-forming virus (SFFV) in Friend and Rauscher virus preparations is an entity distinct from the more typical MuLV's also present in these virus stocks, and that SFFV is defective and dependent on its associated MuLV for some still poorly understood helper function(s). Awareness of this complexity in F-MuLV and R-MuLV preparations should improve the interpretation of data obtained with these viruses, as was achieved previously by awareness of the complexity of defective sarcoma virus preparations.

The existence of SFFV in Friend virus preparations was described in recent reviews on host genes which control MuLV expression (*15, 16*). Emphasized here are the biologic properties of SFFV compared with another more thoroughly characterized RNA tumor virus of mice, murine sarcoma virus (MuSV). Unless otherwise identified, SFFV will refer to Friend (F) and Rauscher (R) isolates, and the presence of MuLV helper can be assumed.

SIMILARITIES BETWEEN SFFV AND MuSV

Like MuSV (*17*), but in contrast to all other MuLV's, SFFV rapidly induces discrete foci of transformed cells, and the number of foci is related to the dose of virus administered (*10, 11*). The standard 9-day assay based on this phenomenon involves the counting of foci in the red pulp of the spleen which have become sufficiently enlarged to be detected at the splenic surface with the unaided eye. Some foci in the spleen undoubtedly escape this kind of detection as do foci that arise in the bone marrow (*18*), but the directly proportional ("one-hit") dose-response relationship observed with spleen focus formation in susceptible mice (*see* below) implies that the frequency of undetected foci remains relatively constant. The unit of virus titer for both MuSV and SFFV is the focus-forming unit (FFU)—that amount of virus required to induce an average of one focus per scoring unit (petri dish or spleen, respectively).

Titrations of SFFV in susceptible mouse strains generally give one-hit dose-response patterns (text-fig. 1B, *broken line*) where the number of foci is directly proportional to the virus dose. From this and many similar titrations, it appears that SFFV is the only *limiting* infectious entity, a conclusion in keeping with the observation that the titer of its helper is normally much higher than that of SFFV itself (*19*). However, in mice restrictive to helper virus expression because of a resistance gene called *Fv-1* (*16*), the dose-response patterns for SFFV expression are "multiple-hit" or "two-hit" (text-fig. 1A, *solid line*) (*12–14*). The *Fv-1* gene restricts MuLV expression ≈10^3-fold near its dilution endpoint (*16*) to a level near that of SFFV. Under these conditions, the multiple-hit patterns reflect a requirement for infection of the *Fv-1*-restrictive cell by at least one (perhaps two) helper virus particle(s) in addition to SFFV for spleen focus formation to occur. A similar requirement for cell infection by a helper MuLV in addition to SFFV may exist for spleen focus formation in *Fv-1*-susceptible mice, but we cannot be certain of this because of the presence of infectious helper virus in great excess. Thus the defectiveness of SFFV was demonstrated by the restriction of the expression of its helper virus with the aid of the *Fv-1* gene.

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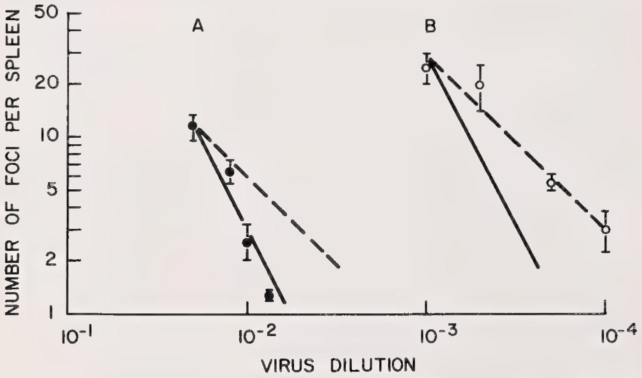
Editor's note: Periodically the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

MuSV is also defective for focus formation (*17*), but the helper MuLV's native to most MuSV strains [Moloney (*20*), Harvey (*21*), and Kirsten (*22*)] are not restricted by either allele of the *Fv-1* gene (i.e., they are NB-tropic); the defectiveness of MuSV was demonstrated in another way. Foci of piled-up round and spindle-shaped cells induced by MuSV in monolayers of mouse embryo fibroblasts form as a result of virus spread (dependent on coinfection with MuLV) and cell division (independent of coinfection with MuLV). The former mechanism predominated in titrations based on focus counts scored only 3–4 days post infection, whereas the latter mechanism became significant in titrations scored 7 days post infection (*23*). Because foci scored early gave two-hit titration patterns and those scored later gave one-hit patterns, it was concluded that MuSV is defective for virus replication but not for cell transformation (*23*). Similar conclusions were reached for Friend SFFV (*24*) when the generation of tumor cells in the spleens of SFFV-infected, *Fv-1*-restrictive mice followed a one-hit dose-response pattern, whereas the generation of infectious SFFV in the same mice followed a two-hit dose-response pattern.



TEXT-FIGURE 1.—One- and two-hit dose-response titration patterns of SFFV in *Fv-1*-restrictive (A) and *Fv-1*-permissive (B) strains of mice. *Broken lines* represent theoretical one-hit titration patterns (number of foci directly proportional to virus dose); *solid lines* are two-hit (number of foci proportional to the square of the virus dose). Experimental data (means ± standard errors, 7 mice per group) are shown.

The helper agents in SFFV and MuSV preparations are MuLV's. Early investigations on the isolation of MuLV from SFFV-containing preparations were arduous, for the SFFV had to be removed by serial passage in resistant hosts, i.e., rats or C57BL mice (*19*, *25–27*). Later, advantage was taken of the knowledge that MuLV's can be isolated from MuSV preparations by dilution past the endpoint for focus formation (*17*, *28*) or sarcoma induction (*29*), and similar studies showed that Friend virus preparations also contain a lymphatic leukemia-inducing MuLV (*19*, *30*, *31*).

Further evidence of the defectiveness of SFFV and MuSV is provided by "rescue" experiments on established lines of cells transformed by these viruses but with no evidence of virus release. Infection of MuSV-induced tumor cells with helper MuLV results in rescue of the MuSV genome as infectious virus. The MuSV(MuLV) pseudotype recovered has the genome (pathogenic) properties of MuSV and the envelope (host range and antigenic) properties of the MuLV helper used to rescue it (*32–34*). Initial experiments with a cell line apparently transformed by Friend SFFV cannot be defined as precisely because the classic parameters of Friend virus disease (splenomegaly and erythropoiesis) were used instead of direct measurements of SFFV. However, Moloney and Graffi MuLV's were successful as helper viruses in retrieving what was probably infectious SFFV from a noninfectious, SFFV-induced tumor (*35*, *36*). This was confirmed during a recent immunologic study (*37*) in which Friend SFFV was rescued from another nonproducing, SFFV-induced tumor after it had been coinfected with Moloney MuLV or with the native MuLV (called lymphatic leukemia virus-Friend, or LLV-F) in Friend virus preparations. The viruses recovered are presumed pseudotypes of SFFV based on host-range experiments (*38*). Both host-range (*14*) and antigenic (*39*) analyses confirmed the effectiveness of the alternative way of making Friend SFFV(MuLV) pseudotypes—by serial passage of SFFV and a B- or NB-tropic helper MuLV in hosts *Fv-1* restrictive for SFFV's native (N-tropic) helper virus.

Friend SFFV has recently been compared with all three strains of MuSV in terms of their RNA components (*40*). The heat-dissociated 50–70S RNA of each virus was resolved electrophoretically into two components, the larger of which had the same electrophoretic mobility as the RNA of Kirsten or Moloney MuLV helper. The smaller component, not present in MuLV's, is thought to represent specific genetic information of these replication-defective transforming viruses. Data in conflict with this conclusion were obtained in an even more recent study (*41*) limited to the Moloney strains of MuSV and MuLV. Thus the possible similarity between the RNA components of SFFV and MuSV must remain tentative until confirmed or refuted.

DIFFERENCES BETWEEN SFFV AND MuSV

The similarities between SFFV and MuSV are remarkable, but they have many important distinguishing features, of which the most obvious is their different pathogenicities. MuSV induces sarcomas in infected mice by transforming fibroblasts (*42*) and also by evoking a proliferative host response (*43*); the tumors often regress in immunologically competent hosts (*44*). Because fibroblasts from mouse embryos can be grown easily in tissue culture, where they (or cell lines established from them) support the replication of MuSV and are rapidly transformed by it, this virus-cell system has been intensively studied in vitro (*17*, *23*, *28*, *33*, *41*, *45*). In contrast, SFFV, which replicates in and transforms primitive hematopoietic cells in vivo (*46–49*), grows to such a limited extent in mouse embryo fibroblasts that it is uually lost after three or four passages, and it does not transform these cells at all. In fact, no transformation assay has yet been developed for SFFV in vitro, probably because primitive erythroid cells could not, until recently, be grown and quantitated successfully in tissue culture (*50*, *51*). Consequently, studies on the early interactions between SFFV and its target cells have been largely confined to in vivo experiments. This has often made it difficult to distinguish the pathogenic effects of SFFV from those of its helper virus(es).

Despite this problem, there seems to be little doubt that SFFV induces the rapid erythroleukemic syndrome characteristic of mice infected with Friend and Rauscher virus preparations. Helper virus preparations cleared of SFFV regularly lack the capacity to induce erythroleukemia (Steeves RA: Unpublished observations), and the dilution endpoint for Friend SFFV coincides with the endpoints for the induction of splenomegaly (*52*) and polycythemia (*53*). In addition, SFFV-induced tumors cultivated in vitro retain their capacity for erythroid differentiation (*37*, *54–56*) and either continue to release infectious SFFV (*55*, *56*) or retain its genome in a "rescuable" form (*35*, *37*). Any doubts that SFFV induces erythroleukemia could be based on the probability that SFFV and its helper virus(es) are symbiotic and on the possibility (though unlikely) that helper virus is qualitatively different in its pathogenic capacity at titers higher than those regularly observed in helper virus stocks free of detectable SFFV.

Spleen foci might, therefore, be viewed as multifocal sites of origin of erythroleukemic cells. Although the bone marrow is also involved very early in the disease (*18*), the continuing cell invasion and erythropoiesis are confined principally to the spleen and liver (*6*, *8*), provoking severe enlargement of these organs. The blood is characterized by marked erythroblastosis and lymphocytosis, and, during the terminal stage of the disease, leukemic cells disseminate throughout the host, especially to the kidneys and lungs (*8*).

Though SFFV induces erythroleukemia, it does not always induce polycythemia (*53*). Some strains of Friend virus complex (called FV-P) contained high titers of SFFV and induced polycythemia vera 3 to 4 weeks post infection; other strains (called FV-A) contained less SFFV and induced anemia, sometimes after many weeks. Rauscher virus preparations, which usually contained less SFFV than our Friend virus preparations, regularly induced anemia. Thus the apparent association between SFFV titer and polycythemia has to do not with the input dose of SFFV but with the titer of SFFV ultimately reached in the infected host, and this in turn depends on virus and host factors not yet clearly understood. We will return to the problem of polycythemia later.

The term "erythroleukemia" has been used because the erythropoietic disease induced by the Friend and Rauscher strains of SFFV has a more malignant character than the erythroblastosis described in mice infected with the Harvey and Kirsten strains of MuSV (*21*, *22*, *57*, *58*). The capacity to induce spleen foci is unique to SFFV-containing preparations but is lacking in MuSV preparations unless SFFV is present, as it might be in Friend or Rauscher pseudotypes of MuSV. The development of these foci and the associated erythroid response are independent of the hormone erythropoietin (*59*, *60*), which is involved in feedback control of *normal* erythropoiesis.

Most importantly, cells in spleen foci can grow as tumor colonies in the spleens of unirradiated mice (*61*, *62*). This is compelling evidence that SFFV transforms hematopoietic cells into neoplastic cells. However, one property generally assigned to neoplastic cells is lacking in most, or perhaps all, cells immediately after transformation by SFFV: the property of autonomous growth.

The rapidity with which SFFV transforms cells is unique among MuLV's, and it may even exceed the transformation rate of MuSV. Cells that grow as transplants in the spleens of intravenously inoculated mice have been detected just 2 days after SFFV infection with the spleen colony assay (*62*), and 3 hours post infection with a radioisotope (^{125} iododeoxyuridine) assay (*63*). Although such "early Friend cells" demonstrate a greater proliferative potential than hematopoietic stem cells from normal mice (*62–64*), their capacity for self-renewal eventually declines (*62*), in contrast to the truly autonomous nature of established tumor cell lines initiated from mice surviving 1–2 months post infection (*65–67*). Also, the karyotypes of early Friend cells are indistinguishable from those of normal mouse cells (*68*), whereas cell lines established from late survivors are aneuploid, with up to two similar (*55*) or dissimilar (*68*) metacentric chromosomes. Thus there are at least two kinds of SFFV-transformed cells compared with normal hematopoietic colony-forming cells (CFC) in table 1: the differentiating tumor CFC and the autonomous tumor CFC. The former is the dominant cell in early Friend disease, whereas the latter is either rare or absent, and gradually arises from the former, perhaps by tumor "progression" (*69*).

An assay for the transforming effect of MuSV was developed in tissue culture (*23*), in which the transformation frequency was 1.4% under optimal conditions. Unfortunately, the transforming capacities of MuSV and SFFV cannot be compared directly, because a transformation assay for the latter does not exist in vitro, and tumors induced by the former in vivo are highly immunogenic (*44*).

Among SFFV-transformed cells, those that express themselves as tumor colony-forming units (TCFU) increase 50-fold in the spleen and bone marrow of susceptible mice between 2 and 4 days after intravenous infection with SFFV (*62*). Even with a high dose of virus (e.g., 6,000 FFU) in the inoculum, many uninfected cells would still be available after 2 or 3 days for infection and transformation by progeny SFFV, usually recoverable from the plasma within 3 days of infection at levels equivalent to the input dose (*74*). Whereas this mechanism may account for some of the increase in TCFU, it is also due to continued growth by self-renewal of the initially transformed cells, as demonstrated by the growth of TCFU on serial transplantation in histocompatible but SFFV-resistant hosts (*62*).

The growth kinetics of TCFU in SFFV-resistant mice have also revealed useful information about later phases of the disease. The total number of TCFU/spleen increased exponentially for the first 10 days after transplantation, but diminished to 10^{-2} of the maximum level during the next 10 days (*62*). However, during this second 10-day period, the spleens continued to increase in total cellularity and the mice developed polycythemia. During the next 30 days, the incidence of splenomegaly and polycythemia declined, as demonstrated by graded numbers of transplanted cells in a similar experiment (table 2). Recent studies indicate that the kind of regression described here [not to be confused with regression in certain Friend SFFV-infected mice (*75–77*)] could have been promoted by the greatly reduced survival time of infected erythrocytes (*78*) and by massive death of less differentiated cells (*79*), possibly due to an autoimmune response (*80*).

This sequential development and regression of TCFU, spleen enlargement, and then polycythemia in tumor-grafted but SFFV-resistant mice confirm a much earlier impression (*8*) of Friend virus disease as a virus-conditioned, differentiating neoplasm.

The use of virus-resistant mice crucial to the TCFU assay (*61*) is based on the dominance of the *Fv-1* resistance gene in F_1 hybrid crosses between mouse strains with the *Fv-1*$^{n/n}$ and *Fv-1*$^{b/b}$ genes (*16*). Small amounts of N-tropic SFFV carried over with the

TABLE 1.—*Comparison among classes of primitive hematopoietic CFC, uninfected or infected with Friend SFFV*

Property	Normal CFC (*64*)	SFFV-induced differentiating tumor CFC (*61–63*)	SFFV-induced autonomous tumor CFC (*70, 71*)
Time detectable (after SFFV infection)	Before and after	After 2 days	Rare until 1–2 mo.
Transplantable in irradiated hosts	Yes	Yes	Yes.
Transplantable in unirradiated hosts	No[a]	Yes	Yes.
Evidence of hybrid resistance [b]	Yes	Enhanced	Enhanced.
Evidence of "decline" [c]	Yes	Yes	No.
Karyotype (*55, 68*)	Normal	Normal	Aneuploid.
Probability of differentiating progeny cells	High	High	Low.[d]
Radioprotective effect (*72*)	Good	Fair	Unknown.
Erythropoietin-dependence (*59, 60*)	Yes	No	Unknown.
Friend-Moloney-Rauscher antigen	No	Yes	Variable.

[a] Except in certain genetically anemic mouse strains (*73*).

[b] Hybrid resistance refers to the reduced colony-forming efficiency of CFC transplanted into F_1 hybrids between the donor strain and certain other mouse strains (*71*).

[c] Decline refers to a gradual loss of colony-forming ability after 3–4 serial transplantations in irradiated syngeneic hosts (*64*).

[d] Unless stimulated by dimethyl sulfoxide (*54*).

TABLE 2.—*Clinical course of disease in (SIM × C57BL)F_1 mice after grafting with graded doses of SFFV-transformed tumor cells* [a]

Cell dose	Incidence of splenomegaly[b] (%)			Incidence of polycythemia [c] (%)			Mortality (%)
	2 wk	3 wk	7 wk	2 wk	3 wk	7 wk	9 mo
10^7	100	100	92	93	93	69	64
3×10^6	100	100	100	86	93	50	54
10^6	80	80	60	73	80	20	20
3×10^5	71	71	29	36	43	21	7
10^5	40	14	0	0	21	7	13
3×10^4	0	0	0	0	0	0	0

[a] Groups of 14 or 15 F_1 mice were given washed spleen cells intravenously from SIM mice infected 8 days before with Friend SFFV (4,000 FFU).

[b] Splenomegaly, determined by palpation, was considered positive only when the spleens extended to the midline of the abdomen.

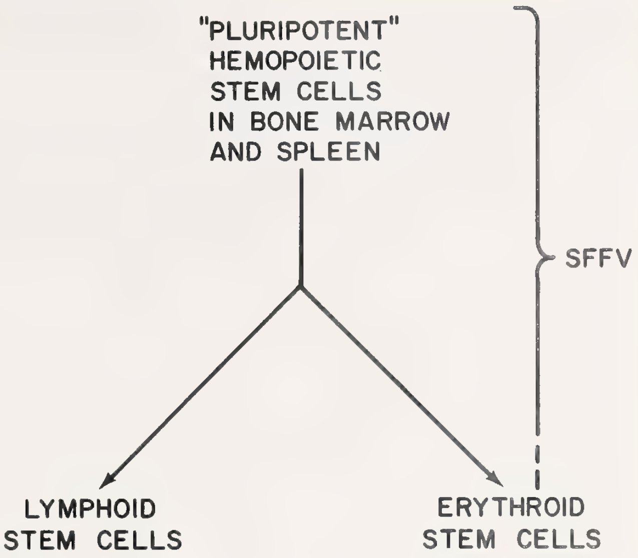
[c] Polycythemia was diagnosed when hematocrits were greater than 56%.

washed leukemia cells from *Fv-1*$^{n/n}$ donor mice have little opportunity to obscure the enumeration of transplanted tumor colonies by the induction of macroscopically similar foci of transformed host cells. However, with the exception of one report (*13*), all Rauscher strains of SFFV are NB-tropic, and are, therefore, not suitable for the analysis of virus-induced TCFU in nonirradiated mice. Lethally irradiated hosts prevent SFFV expression (*10*), but they do allow the expression of any normal CFC's as well as tumor CFC's present in mice soon after Rauscher SFFV infection. According to Brommer (*49*), however, the former are in the minority, as demonstrated with absorbed Rauscher antisera from rabbits, and, with this limitation in the irradiated host assay, Rauscher SFFV-induced TCFU increased rapidly in the spleen after infection (*49*, *81*).

Perhaps because SFFV transforms different cells than MuSV does, SFFV is a potent immunodepressive agent (*82*, *83*), and this may contribute to its rapid lethality in adult mice, also in contrast with MuSV. Whereas the helper MuLV in both MuSV and SFFV preparations is immunodepressive by itself, its effects are only transient and not as profound as those of preparations containing SFFV (*19*). It is quite possible, however, that *a*) agents other than SFFV but associated with it are immunodepressive, *b*) SFFV depresses the host's immune system by one mechanism and stimulates erythropoiesis by yet another, or *c*) one mechanism suffices to initiate both effects of SFFV.

The last hypothesis is compatible with one of the five possible mechanisms for SFFV-induced immunodepression, the common stem-cell model, described by Dent (*82*). If we view the "pluripotent" stem cell of the bone marrow (*84*) as a precursor of both lymphoid and erythroid stem cells (text-fig. 2), we might allow SFFV, by the infection of one or several stages of differentiating cells, to cause a significant increase in the fraction of cells entering the erythroid pathway at the expense of the fraction of cells entering the lymphoid pathway. As more and more pluripotent stem cells or their progeny become infected by SFFV in rising titer and as they become committed to erythropoiesis, certain pools of cells along the lymphoid pathway to the B cell and plasma cell become severely depleted, and humoral immunity in the host is compromised (*85*).

Two interesting observations also fit this model. First, hyperstimulation of the immune system results in diminished sensitivity to Rauscher SFFV, possibly by exhausting the lymphoid reserves in the bone marrow and reducing potential target cell pools for the virus (*86*). Second, a single dose of endotoxin given to mice 4 days before Friend SFFV increases their sensitivity to the virus (*87*), apparently by triggering the release of a "cohort" of potential target cells from the pluripotent stem-cell pool (cells which, without SFFV infection, might have been destined for the lymphoid pathway). Future experiments that take advantage of our growing understanding of immune mechanisms will help to refute or extend this hypothesis.



TEXT-FIGURE 2.—SFFV may infect one or more stages of cells differentiating from the pluripotent stem-cell pool and down the erythroid pathway, thereby causing a shift toward erythropoiesis and away from lymphopoiesis to the B (bursal-equivalent) cell. Any effects of SFFV-containing preparations on T (thymus)-cell function could be caused by helper MuLV.

Still another difference between SFFV and MuSV is the genetic control of host resistance and susceptibility to these viruses. Though the diseases induced by either virus can be influenced by an *H-2*-associated gene and the *Fv-1* gene, the *Fv-2* gene is specific for the expression of SFFV (*16*). Mice homozygous for the recessive resistance allele of *Fv-2* are completely refractory to spleen focus formation by SFFV, though they will support the replication of its helper MuLV (*19*), and even, to a limited extent, of SFFV itself (Steeves RA, Lilly F: Unpublished observations). This indicates that *Fv-2*$^{r/r}$ cells can be infected in vivo, in keeping with their lack of resistance to infection in tissue culture (*16*), so the *Fv-2* gene must inhibit cell transformation or TCFU growth to prevent spleen focus formation. The latter alternative was supported in a recent study (*88*) which suggested that host cell rejection of TCFU might be involved.

Finally, SFFV and MuSV differ with respect to the specificity of agents which will provide a helper function for them. In addition to MuLV's, which help both viruses, feline leukemia virus (*89*) and similar viruses from hamsters (*90*) and rats (*91*) will increase the focus-forming efficiency of MuSV in tissue culture; this requires the use of nonmurine cells, however, usually cells of the same species from which the helper virus was isolated. In contrast, with the in vivo assay for helpers of SFFV (*92*), the host species could not be changed without losing sensitivity to SFFV completely, but despite this requirement, helper activity was detected among an even broader range of samples, including extracts of human leukemic spleens (*93*), mycoplasmas (*94*), and several species of bacteria (*95*). The reason for this difference is not clear. Though it may have to do with the fact that the assay for SFFV is conducted in vivo, there is no evidence that any of the nonviral helper factors depress the immune system of the host or stimulate its target cells (*95*).

Many virus stocks containing SFFV or MuSV also contain the lactic dehydrogenase-elevating virus (LDV) (*96*). Whereas LDV can potentiate the oncogenic effects of both viruses (*87*, *97*), these effects were originally observed only when LDV infection preceded infection with SFFV or MuSV by 2–3 days, and they were thought to be mediated by stimulating target cells for the virus (*87*) or by depressing the host's cellular immunity (*97*). LDV did not provide helper activity for SFFV in the standard helper assay (*92*). In more recent studies, however, removal of LDV from a Rauscher virus preparation reduced its pathogenicity significantly, and replacement of LDV into the "uncontaminated" Rauscher virus pool restored its activity (*98*, *99*). This does not exclude the possibility of a target cell or immunodepressive effect of LDV, but it does raise again the question of the possible role of LDV as a helper virus for SFFV.

RELATIONSHIP BETWEEN FRIEND SFFV AND RAUSCHER SFFV

Though there is little doubt that the antigenicities of their native helper MuLV's are distinct from one another (*39*, *100*), no precise marker has yet been used to compare the SFFV in the Friend and Rauscher virus complexes. The Rauscher virus complex differs from most of the Friend strains by not inducing polycythemia and by inducing extensive splenic necrosis and the development of blood-filled sacs in the spleen in susceptible mouse strains. These differences do not seem related to differences in SFFV titer, and the two strain designations should probably be retained, perhaps also for historical reasons, until a detailed comparison of the SFFV genome in each preparation becomes feasible.

TERMINOLOGY PROPOSED

To be aware of the complexity of Friend and Rauscher leukemia virus preparations is admirable and relatively easy, but to communicate effectively by the clear identification of the particular virus in the complex under consideration is necessary and often difficult without a logical terminology. Among the defective RNA tumor viruses, terminology has been most successful in the avian system (*5*), except, perhaps, for avian sarcoma virus itself (usually abbreviated RSV rather than AvSV). Here, the memory of Peyton Rous is indelibly imprinted on our minds. In the murine system, the term MuSV was gradually accepted, once several strains [Moloney (M), Harvey (H), and Kirsten (Ki)] came into common use. How logical it is, then, to write Friend SFFV (F-SFFV) and Rauscher SFFV (R-SFFV), just as one writes Schmidt-Ruppin RSV (SR-RSV) or Harvey MuSV (H-MuSV), with the helper virus assumed to be the native one unless indicated otherwise (table 3). If SFFV is not present in the preparation (e.g., after serial passage in mouse fibroblasts in culture), one simply writes Friend MuLV (MuLV-F) or Rauscher MuLV (MuLV-R), just as Moloney MuLV (MuLV-M) and Kirsten MuLV (MuLV-Ki) are in use already. The term LLV originally used for the helper viruses in Friend (*25*) and Rauscher (*26*) virus preparations is no longer needed once the distinction between SFFV and MuLV is made. The only fly in the ointment is the unfortunate alliteration that occasionally leads to flies in the mouth when SFFV is spelled aloud during rapid conversation. Alas, SFV is already used to designate Semliki forest virus and simian foamy virus.

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Table 3.—*Terminology and abbreviations proposed for the Friend and Rauscher virus complexes and their components*

Common name [a]	Proposed name	Proposed full abbreviation
Friend virus (complex)	Friend SFFV	F-SFFV(MuLV-F)
Rauscher virus (complex)	Rauscher SFFV	R-SFFV(MuLV-R)
Friend (helper) virus	Friend MuLV	MuLV-F
Rauscher (helper) virus	Rauscher MuLV	MuLV-R
Moloney pseudotype of Friend virus	Moloney pseudotype of Friend SFFV	F-SFFV(MuLV-M)

[a] Words *in parentheses* have been added in this column for the sake of clarity; their usual omission is perhaps the greatest cause of communication problems in this field today.

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ADDENDUM

Dogmas either stand the test of time or they just fade away, like old soldiers. The 4 years since this Editorial was written have been sufficient to see some of its themes considerably supported and extended as others are rapidly fading from view.

The concept that SFFV is a unique, replication-defective MuLV that causes the rapid transformation of certain hematopoietic target cells has gained acceptance as a result of several studies, most notably those of Troxler et al. (*1–3*). To analyze the gene(s) of SFFV that might be responsible for its leukemogenicity, they first isolated SFFV free of replicating helper virus in nonproducer fibroblast cell lines (*1, 2*). Using these cell lines in molecular hybridization studies, they demonstrated that SFFV was a recombinant between a portion of ecotropic helper virus (Friend MuLV) and a portion of mouse xentropic virus (*2*). Then in an extension of this work, they showed that a complementary DNA that represented a xenotropic virus portion of SFFV also detected genetic sequences derived from the *env* (envelope) gene region of murine xentropic virus (*3*). Whether the xenotropic viral sequences in SFFV are responsible for its rapid leukemogenicity remains to be determined.

An area of continuing controversy is the basis for which the term "erythroleukemia" is used to characterize the disease induced by SFFV, although "erythroblastosis" may more aptly reflect the erythropoietic stimulation induced by Kirsten MuLV. Two criteria were presented for erythroleukemia in this review: erythropoietin-independent erythropoiesis and the growth of SFFV-transformed cells into spleen colonies in *Fv-1*-restrictive hosts. Whereas the first criterion still applies, the spleen colony assay is no longer considered a valid measure of the proliferative capacity of SFFV-transformed cells. Rather, spleen colony formation in *Fv-1*-restrictive but unirradiated hosts was recently shown to be based on host (rather than on donor) cell proliferation (*4*). Therefore, the spleen colony assay for Friend leukemia cells in unirradiated hosts is an assay for infectious centers where virus is apparently released from donor cells in quantities sufficient to infect *Fv-1*-restrictive host cells and to initiate their proliferation.

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GUEST EDITORIAL — **by L. M. Shabad, M.D.**

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Precancerous Morphologic Lesions

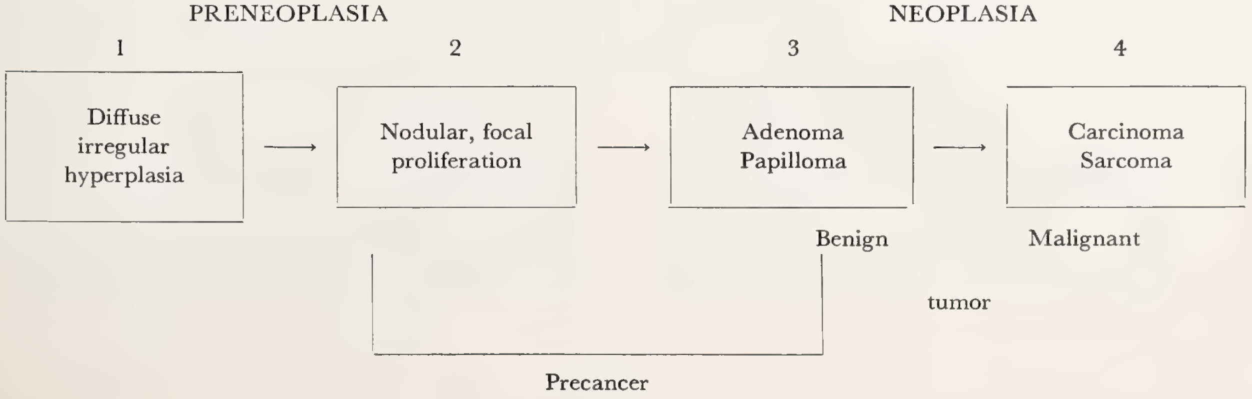
THE CONCEPT OF PRECANCER has not been studied sufficiently and some aspects remain disputable, especially in regard to the morphology and histologic definition of precancerous lesions.

The concept of precancer and the term itself have been discussed many times and provoked fierce arguments. Clinicians accepted the idea of precancer, but included all the processes and diseases preceding cancer. Pathologists denied the concept of precancer and considered it impossible to prove the transition of a morphologic cancer precursor into a malignant tumor. They believed "cancerous" or "noncancerous" lesions could be identified only by microscopic study.

Morphologic evaluation of the precancerous character of any lesion is difficult. If the pathologist finds such cancerous features in the tissue specimen as invasive and destructive growth, he decides that this is a cancer. If such features are absent, he cannot be sure that the lesions would lead to cancer because he is studying only a separate frame. Furthermore, the examined lesions cannot develop into cancer because they are already eliminated from the organism by biopsy or surgery.

Another difficulty lies in the differentiation of many background, nonspecific processes from the true precancerous or pretumorous lesions. A serious problem is to evaluate true precancerous lesions, which are a definite stage in the development of cancer, and to distinguish them from unspecific lesions, which are not necessary for the initiation and development of tumors.

Despite all the problems, the concept of precancerous conditions is well established in clinical practice, especially for tumors of the skin, oral cavity, stomach, bowel, mammary glands, uterus, and bladder. The basic reason for this assertion is that none of the well-studied tumors arose suddenly and "ex vacuo." Each was preceded by definite morphologic changes that developed over a long time. Malignant tumors are the last link of a long chain of pathologic events, which can be called pretumorous and precancerous. We proposed the term "pretumorous" or "preneoplastic" for the early changes preceding benign neoplasia and the term "precancerous" for those immediately transiting to malignancy (*see* chart below).



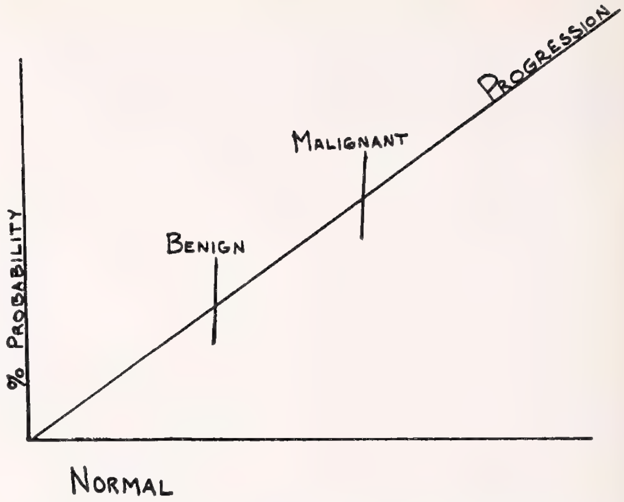
Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

The term "precancer" is accepted by the World Health Organization [*see* Technical Report No. 276 of the Expert Committee on the Prevention of Cancer (1964), section entitled "Precancer" (*1*)].

At present, malignant tumors of any type and localization can be experimentally induced by chemical carcinogens and radiation, but only the experimental-morphologic investigations allow systematic study of the step-by-step development of malignant tumors in a variety of materials. Results obtained from this type of study contributed to the elucidation of the concept of precancer. Without exaggeration, only such experiments gave a real meaning to the concept of precancer.

For many years, my colleagues and I have studied precancerous lesions in animals and man; the results of these studies were reviewed in (*2*). Also described were experimentally induced precancerous changes of the skin, lungs, stomach, pancreas, liver, kidneys, mammary glands, uterus, ovaries, testes, prostata, seminal vesicles, thyroid gland, and presarcomatous lesions. These experimental results gave an impetus for a thorough investigation of precancerous lesions in man. We have compared them, especially the human precancerous lesions of the mammary glands, prostata, bladder (both occupational and "spontaneous" precancer), skin, larynx, stomach, bowel, kidneys, and some other organs and tissues. The results of the experiments were evaluated from the clinicopathologic point of view and vice versa.

From the clinical point of view, "precancerous" is defined as clinical conditions preceding cancer with an increasing probability of transforming into cancer (text-fig. 1). From the morphologic point of view, "precancerous" is defined as established gross and microscopic changes that can be evaluated as the regular beginning of cancer from the precancerous lesions. Some of these changes, like Bowen's dermatosis and xeroderma pigmentosum, always transform into cancer and are considered *obligate precancer*. In others, like some forms of mastopathy or cervix erosion, the beginning of malignancy is observed in certain forms and at certain stages of the process, but occurs only in a definite percentage of the cases. Such lesions are called *facultative precancer*. However, the whole concept of precancer is facultative: In other words, precancer can develop into cancer, but it does not always do so. Finally, in the variety of diseases, lesions, and changes that precede the cancer, one must distinguish those influencing its development and those having no connection with it. The first can be estimated as the *background of precancer*.



TEXT-FIGURE 1.—Progression of precancerous lesions to malignancy.

Morphologically, we can identify various precancerous changes even in the same organ. Examples are: *kidneys*, focal growth of canalicular or glomerular capsular epithelium that is the source of further adenoma and adenocarcinoma development, or the growth of pelvic epithelium that forms an epidermoid carcinoma; *mammary glands*, precancerous growth of ductal and acinal epithelium; and *lungs*, bronchial and alveolar epithelium. The structure, histogenesis, and characteristics of the precancerous lesions determine the characteristics of the arising tumors. For example, in the bones, nodular proliferation of fibroblastic, chondroid, or osteoid tissue was observed and osteogenic sarcomas of different features consequently arose.

More pronounced differences in the morphology and development of precancerous lesions can be detected in different organs. The differences between precancer of the skin (or of the liver) in man and in experimental animals are considerably less than those between precancer of the skin and the liver in the same species. These peculiarities depend on the special characteristics of each organ and tissue and on their metabolism. Precancerous lesions of different organs and tissues are no less, and even more, different than the developing tumors—they represent different nosologic entities, different diseases. At the same time, the general and common features of the pathologic process of cancer development are similar and are reflected in each nosologic unit. Therefore, some common features can be discovered in the precancerous lesions of different organs.

Each cancer has its own precancer. But each precancer does not develop into cancer.

The origin of cancer is often multicentric. Accordingly, precancerous lesions are usually multiple. As a rule, the number of such lesions is greater than the number of tumors developing from them. The multicentricity of the tumor initiation is easily established by the experimental investigation of each form and each localization of tumors—the skin, subcutaneous tissue, lungs, mammary glands, liver, stomach, uterus, ovaries, etc. Thus, for example, when the skin of mice is painted with carcinogenic tars and hydrocarbons, numerous papillomas arise at the site of application and on remote areas of the skin. These proliferations do not appear at the same time and they develop at various speeds. As a result, multiple and different nodules and stages of tumor development can be observed at one time and even in one microscopic slide. Some multicentric, epidermoid, papillary epitheliomas can be induced in the vagina and cervix of mice by intravaginal introduction of tampons with 7,12-dimethylbenz[*a*]anthracene (DMBA). The multiplicity of the experimental neoplasms can be observed in all organs and tissues.

The same multicentricity of the foci of precancer is observed in human pathology. This can be verified by slides showing multiple hepatomas. The multiplicity of stomach and bowel adenomatous polyps is well known. The multiplicity of precancerous lesions also is seen in other organs, such as mammary glands, ovaries, and kidneys.

From our investigations, we can distinguish two types of precancerous lesions: 1) the precancers of covering epithelium and 2) the precancers of the secretory epithelium.

There are many common features in precancerous lesions of the skin and mucosa of the lip, oral cavity, esophagus, prestomach in mice and rats, and vagina. Since epithelial growth and papillomas in these organs and tissues in areas in which carcinogenic substances were applied are similar to reactive growths and inflammatory lesions, these two different processes are not easily distinguished. Examples are the focal enlargement and atypical growth of the skin epithelium in Bowen's disease in man and the focal proliferation of skin painted with DMBA in mice. We have observed the focal growth of immature fibroblasts during sarcoma development. These fibroblasts can be evaluated as presarcomatous nodules.

On the other hand, some similarity can be found in the precancerous changes of such parenchymatous organs as the liver and kidneys. In both, the first stage of the process is diffuse, irregular hyperplasia, then focal proliferations, and, finally, adenomas. In both organs, precancerous changes of the parenchyme and ducts can be distinguished. In the kidneys, as in the liver, the precancerous changes usually arise far from the site of carcinogen application and they can be easily distinguished from inflammatory lesions. The irregular, diffuse, and focal hyperplastic growth of the epithelium of the Bowman's capsules and of the tubules of kidney of male hamsters treated by estrogens is an example. In man some early stages of growth of kidney adenomas are seen. Some differences in the type of precancerous changes in various organs with secreting parenchymas have been noted. These depend on the different structure and function of the organs and on the peculiarities of the pathogenesis of tumors. It is sufficient to compare the precancerous changes of ovaries, prostata, thyroids, and mammary glands. The precancerous changes of these organs and tissues have some common features, the most important of which is that the first stages of their development are difficult to separate from physiologic changes. This difficulty can be overcome if we remember that cancer development is a pathologic process; therefore, we can consider as precancerous or pretumorous only those changes that are out from the normal physiologic framework.

I believe all the forms of precancerous changes can be divided into two groups: 1) those occurring near the first application of carcinogenic substances in the tissues and organs directly exposed or "open" to the influence of environmental agents, and 2) those occurring far from these areas in the tissues and organs shielded from the environmental agents.

The first group includes precancerous changes of the skin, stomach, and lungs—both in experiments with carcinogenic agents and in human pathology. The second group includes changes induced by aminoazo dyes or fluorenes in the liver, or the lesions of the hypophysis, mammary glands, ovaries, and testicles caused by hormonal disturbance provoked by introduction into the organism of high doses of estrogens or by hypophyseal pathology. The principal difference between the two groups of precancerous changes is the inflammation. In the first group, the inflammation always takes place in the form of the primary reaction to foreign-body or substance introduction. This primary inflammatory reaction can be later changed, depressed, and distorted by carcinogenic substances, as shown by Vasiliev (*3*) in experiments on the subcutaneous introduction of carcinogenic hydrocarbons in paraffin pellets. Saliamon (*4*) also depressed the inflammatory reaction by carcinogenic substances in pathophysiologic experiments. Repeated intratracheal injection of carcinogenic substances in rats provoked repeated inflammation in the lung tissue. This led to the complicated pathologic background from which cancers induced by carcinogenic substances develop. Such changes also can occur in human pathology.

In the origin of dishormonal tumors, the fine and intimate carcinogenic changes in the tissues and cells take place without any rough action on the tissue from foreign irritation, which can be accompanied sometimes by a nonspecific influence, and inflammation does not occur. Numerous experiments in our laboratory showed that inflammation does not promote the development of cancer and is not necessary to its induction. We thus affirm that inflammation is not a necessary link in the chain of events leading to cancer and is not a precancerous condition.

However, in the first group the inflammatory and precancerous components are not easily distinguished, particularly in the first stage of tumor development—diffuse, irregular hyperplasia. The relationship between the inflammatory and the precancerous changes is complicated. It must be considered in connection with different aspects, forms, degrees, characters, stages, and causes of inflammation and also in connection with the origin, development, and progression of tumors. It also should be evaluated individually for different groups, types, and localizations of pretumoral changes (*5*). The presence and the character of the inflammatory reaction during the induction of cancer depend on the properties and dose of the carcinogenic agent and on the route and mode of its administration or penetration into the organism.

Elucidation of the role of inflammation in the origin of tumors is of great clinical significance. Some clinicians still attribute a precancerous role to inflammation, if only for certain forms and localizations of tumors. In many countries, such a view is reflected in the nomenclature of precancerous lesions. In some American textbooks, fibroadenomatosis of the mammary glands was called "mastitis chronica." But at present nobody should consider the adenomatous polyps of the stomach or bowel as inflammatory conditions. There is no doubt about the dishormonal nature of the adenomatous growth in prostata or mammary glands. The same can be presumed for the so-called erosio cervicis. We are approaching the revision of the role of inflammation in the origin and development of some other precancers.

The cancer and the inflammation are quite different pathologic processes, and there is sufficient evidence against a constant precancerous significance of the inflammation. This does not mean that the two processes sometimes do not coincide. For example, in experimental lung-cancer induction in rats, precancerous lesions developed from bronchiectasis with chronic inflammation; but in other instances, they appeared without any connection with inflammatory foci. Therefore, cancer may arise sometimes on the ground of inflammation or from its results (background of precancer), but these facts cannot be regarded as evidence in favor of the precancerous significance of this process.

Morphologically, we may define precancer as microscopic, multicentrically originated, often multiple foci of noninflammatory atypical growth of undifferentiated epithelium (e.g., of fibroblastic or osteoid tissue in presarcomas) that tend to invade but not destroy the surrounding tissues. These relatively "benign" tumors and focal proliferations, which are most often transformed into cancer without any intermediate stages, must be called precancer in the strict sense of this word. The irregular, diffuse hyperplasia, a precursor of focal proliferations, is not precancerous in the strict sense, but can be regarded as a pretumoral preneoplastic lesion (*see* chart, p. 1421).

I believe that the concept of precancer must be more definite and precise. It is better to hear the reproach that the concept of precancer coalesces with the concept of cancer than to permit this first concept to become confused and diluted by the mass of unspecific changes in various previous illnesses. We must designate as precancer only definite early stages and first steps in the development of cancer and not concomitant and unspecific lesions.

The common features of precancer are particularly clear if we examine the four successive stages of the development of cancer: diffuse, irregular hyperplasia, nodular focal proliferation, benign tumors, and malignant tumors. Precancerous nodular proliferation sometimes transforms directly into cancer without forming "benign" tumors. Usually, however, all four stages of the development of cancer can be detected in all the organs and tissues.

Stage I, a peculiar diffuse, irregular hyperplasia, differs from inflammatory and regenerative hyperplasia. This stage is difficult to determine because it is near the normal state and only the beginning of the pathologic condition that becomes more and more specific. Stage I is also hard to differentiate from other types of hyperplasia, e.g., from dishormonal hyperplasia. The pretumoral hyperplasia is abnormal morphologically and functionally. This abnormality is expressed by the inequality of morphologic manifestations and functional state and activity. A striking instance of such inequality is the diffuse hyperplasia of the epithelium of mammary glands induced by large amounts of estrogens in male or castrated female rats. The alveoli of the mammary glands are distended by secretion and are transformed into cysts of various size and form; their content is of different thickness and coloring. The picture is similar to galactostasis. The histologic appearance of such hyperplasia resembles that of the lactating mammary gland, but, of course, true lactation does not occur here. Such a condition can be called a pathologic neoplastic variant of the process of which the lactation hyperplasia is a physiologic prototype.

Sometimes the hyperplasia of the tissue is regular morphologically, but focal changes can be detected by histochemical investigation. For example, in the diffuse hyperplasia of theca-tissue in the ovaries of mammary carcinoma patients, foci with high activity, of oxidation-reduction enzymes and steroid hydrogenase, were found. Thus the atypical form was preceded by atypical function. During the induction of experimental ovarian tumors in mice, analogous, diffuse, irregular hyperplasia was observed.

Further, the diffuse, irregular hyperplasia, e.g., of epithelium of the mammary glands, becomes more and more erratic, and atypical and focal proliferations of the epithelial cells in the cystically enlarged alveoli of the gland are observed. The transition of the pretumoral changes from the first to the second stage takes place.

Stage II is focal growth (nodular proliferation). This proliferation differs from surrounding tissues because of some atypical features of general tissue structure and of cell appearance. Its growth is sometimes infiltrative, but not destructive. Such focal proliferation of the bronchial epithelium—partly adenomatous and partly metaplastic and epidermoid—can be induced in rats by the intratracheal introduction of carcinogenic hydrocarbons (benzo[*a*]pyrene and DMBA) with adsorbents (*6*). Similar focal proliferation of the cervical and vaginal epithelium occurred in experiments with mice treated locally with DMBA tampons. It begins with a slight nodular thickening of the epithelium and develops further to endophytic or to exophytic growth which forms papillomatous tumors.

In human pathology, such focal epithelial proliferation often can be seen in the development of various forms and localizations of cancer, e.g., in the esophagus as well-circumscribed, superficial leukoplakia, in the larynx as endophytic growth of the epithelium by pachydermia, and in the cervix as epidermoid-sinking growth reaching the glands by the so-called nonhealing erosio cervicis.

We demonstrated that such nodular proliferation has its own stroma and increased and peculiar blood supply.

The limit between the focal proliferation and the true "benign" tumors is not easily defined. Nodular proliferation in mammary glands of mice, the so-called adenomatous foci, is, strictly speaking, a microscopic adenoma. On the other hand, especially in human pathology, some forms of focal growth in the mammary glands, e.g., in the cystic mastopathy, can be clearly distinguished from such tumors as the fibroadenoma.

Diffuse, irregular hyperplasia, nodular proliferation, and "benign" tumors are peculiarly interrelated in the human stomach. On the borders of chronic callous ulcer, focal epithelial proliferation of adenomatous character can be detected. The size, form, and staining of such proliferation differ widely from those of the normal mucosa. In the stomach polyps, similar hyperchromic atypical glandular acini have been noted, which we believe indicate eventual malignancy. Sometimes in these glandular acini, epithelial cells conglomerate and pile up. Further, the focal growth of the glandular epithelium can invade the stomach wall.

Stage III of cancer development is represented by "benign" tumors (papillomas, adenomas). Experiments have shown clearly the relativeness of the "benign" nature of such tumors and the real possibility of their becoming malignant. They are only temporarily benign. Of course, not all benign tumors become malignant; many remain benign during the entire life of man or animals. But each benign tumor can become malignant. Carcinoma can arise in the most benign tumor—the fibroadenoma of mammary glands (from nodular focal growth so typical for adenomatous-cystic mastopathy).

These "benign" tumors are particularly common in experimental skin carcinogenesis. Various forms of benign skin tumors are observed, e.g., papillomas, acanthomas, or adenomas of sebaceous glands. The connection of papillomas with some preceding epithelial proliferation is particularly clear in human pathology. In mammary glands, for example, intraductal and intracystic papillomas often arise from adenomatous mastopathy. In the development of ovarian neoplasms, sometimes in the same tumor, the transformation of the diffuse, irregular hyperplasia and the focal proliferation into papillary growth and into intracystic papillomas can be seen. Even in the development of cervical carcinoma, in which endophytic growth predominates, sometimes exophytic papilloma-like growth can be detected. Morphologically, typical papillomas are the precursors of cancer of the bladder, both as an occupational and as a nonoccupational disease. Clinically, bladder papillomas are potentially malignant and clearly demonstrate the indissoluble ties of different stages of the process of cancer development.

The possibility of the transformation of benign tumors into malignant ones must be emphasized because this fact, which is indisputable in our opinion, is still under discussion by other investigators. Ackermann et al. (*7*, *8*) do not believe that benign adenomatous stomach and bowel polyps can be transformed into adenocarcinoma; they consider that malignant polyps are cancerous from the beginning of their growth. Reuber (*9*) doubts that the liver adenomas in corresponding experiments are transformed into malignant hepatomas. According to the already mentioned report of WHO: "In the majority of experiments on epithelial tissues, the induction of benign tumors is only a stage in the process, which leads to malignant growth." In our opinion the "benign" tumors have significantly more chances to be transformed into cancer than corresponding normal tissues.

The adenomatous stomach and bowel polyps are really transformed into cancer. The stage of benign tumors in carcinogenesis is more often observed than described. Specifically, tumorous growths, such as the intraductal and intracystic papillomas, the mammary glands and ovarian adenomas, the adenomas of kidneys and liver, the papillomatous growth of the cervix, and similar neoplasms, are transitory benign tumors—stage III of carcinogenesis. We should not forget the possibility of a direct cancerization of the precancerous nodular proliferation without the formation of the "benign" tumors. Stage IV is already the malignant tumor, the cancer.

The four stages of the development of cancer can be found, as a rule, in all forms of induced cancer which are studied well enough. However, the frontiers between these stages can never be strictly determined because they are converted one into another and are only the various phases of one process.

At present, the concept of tumor progression [*see* Foulds (*10*)] is unanimously adopted. If the already developed malignant tumor does not represent something unvariable, but changes continuously in the direction of the increased malignancy, there is even more reason to apply this concept to precancerous and pretumorous lesions.

The precancerous lesions always "depend" on the organism. It is the basic biologic characteristic of precancer. As soon as the lesion starts to become "independent" (autonomous), it is already a cancer, not a precancer. The benign tumors, which in our opinion are the third stage of carcinogenesis, depend on the organism. Uterine fibromyomas and mammary fibroadenomas are striking examples, which "floruish" and "fade" during a woman's life: in other words, they are directly dependent on the changes of the hormonal state.

Thus tumor progression begins with the progression of the pretumoral growth. The difficulties of precancer determination are not more than those of any other developing pathologic process. Important facts in favor of these ideas are provided by data on the duration of cancer growth. Experimental investigations have firmly estabished that malignant tumors arise from medium-strength carcinogenic agents after a period equal to about one-fifth the longest lifespan, e.g., about 6 months for rats and mice, who live no more than 2.5 years. During this latent period, the precancerous and pretumoral changes are arising. We must emphasize that the latent period in some occupational cancers in man is 15–18 years, on the average; it is also one-fifth the longest lifespan. This is true for cancer in chimney-sweeps, bladder cancer in aniline-dye workers, cancer in radiologists, and others. The same laws are observed also in the development of malignant tumors induced by thorotrast (thorium dioxide)—a radioactive substance formerly used in several countries as a contrast substance in radiology. Thus human malignant tumors, when their cause is known, arise long after the active beginning of such cause. There is no reason to believe that development of the spontaneous tumors in man, i.e., the tumors whose causes are still unknown, is essentially different.

Modern clinical studies, especially radiologic investigations, have demonstrated the long duration of many forms of cancer, e.g., of mammary gland and lung cancer. The long duration of such easily detected cancers as skin cancers and lower lip carcinoma has been known. If the cancer can exist in the organism for some years, the precancerous changes can develop over many years. This has been demonstrated by direct clinical observations on precancerous lesions of skin, cervix, mammary glands, and stomach.

All these observations prove that precancer and cancer develop gradually over a long time, with possible fluctuations. The study of the pretumoral and precancerous changes showed that the specificity of the tumoral process increases gradually, step by step. This is true, not only for the morphologic but also for the physiologic, biochemical, and immunologic particularities.

Different precancerous processes, as well as benign and malignant tumors themselves, can develop at various rates. Papilloma of the skin, induced in mice painted with a small dose of carcinogenic hydrocarbons or with a weakly active hydrocarbon, grows slowly. The reverse is true for highly active carcinogenic compounds applied in large doses. Thus the development of precancerous lesions can be controlled experimentally. These facts must be confronted with clinical observations showing that precancerous changes can stop or be stopped by adequate treatment.

Precancerous changes not only can be stopped in their development, but also can fully disappear. The possibility of disappearance of true precancerous lesions can be proved by careful observations on papillomas of the skin induced in mice with carcinogenic compounds. It is well known that the number of cancers arising on the painted skin is less than the number of papillomas which are their precursors. This depends not only on the confluence of some papillomas but also on their disappearance (their regression). In some precancerous lesions and benign tumors, e.g., in experimental adenomas of the liver in mice, we find necrobiotic, dystrophic, and other regressive changes.

Finally, in some cases we can induce epithelial hyperplasia and focal proliferation that do not transform into tumors, e.g., the prostatic hyperplasia and metaplasia induced in mice by estrogens or the mastopathy-like changes in the mammary glands of mice observed by milk stagnation by forced breeding (repeated pregnancy without lactation).

In such a way, not only the diffuse hyperplastic but also the focal precancerous proliferation can disappear and not progress into cancer. The possibility of such spontaneous "healing" of precancerous lesions is a serious reason for the facultative character of precancer and for the success of its rational treatment. Further study of precancers can form the basis for a new direction in cancer control—chemotherapy and hormonotherapy of precancerous lesions as the way of treatment and prevention of cancer.

Precancer is a dynamic concept and differs from such concepts as "latent cancer" (of the prostata) and "carcinoma in situ" (of the uterine cervix). We cannot agree with the two last terms because of their intrinsic contradiction. If the cancer is "in situ," or "latent," without infiltrative and destructive growth, it is not yet a true cancer. And if it is a cancer, it cannot be "latent" or "in situ." As to the clinical significance of "carcinoma in situ," it is generally accepted (and reflected in some international classifications) that it corresponds to the clinical stage 0. But zero is nothing, and it means that carcinoma in situ is not a carcinoma! We believe "carcinoma in situ" (better term, in our opinion, is "preinvasive stage of cancer") or "latent cancer" are in fact not cancers, but precancers. "Carcinoma in situ" is a condition depending on the organism, *eo ipso* biologically it is rather a precancer than a cancer.

Precancer is a real concept from the point of view of the clinics, the experimentation, morphology, immunology, and perhaps biochemistry. Further investigation of the precancer will lead not only to the elucidation of the pathogenesis of various tumors but also to improvement of the early diagnostics and to the cure of precancerous lesions, that is, to the prevention of cancer.

The detection and cure of precancer are the best ways to prevent the development of cancer and one of the principal ways to cancer control with regard to population, as well as to individual patients.

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ADDENDUM

The evaluation of morphologic precancerous lesions remains a serious theoretical and practical problem still under discussion. During the last 5–6 years, much experimental work, especially with new carcinogens such as the various nitroso compounds, was done. In my opinion, all the statements of my paper of 1973 were confirmed. I must emphasize that the idea "Each cancer has its own precancer" remains true for all kinds of neoplasms, but we must keep in mind that "its own precancer" means that the forms, features, capacities, and morphology of various precancers may be different.

Further investigations are needed to clarify many obscure points of the etiology and pathogenesis of tumors, but the concept of precancer is a real and useful one for the understanding of the development of cancer and for its control and prevention.

GUEST EDITORIAL—by Carel F. Hollander

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Animal Models for Aging and Cancer Research

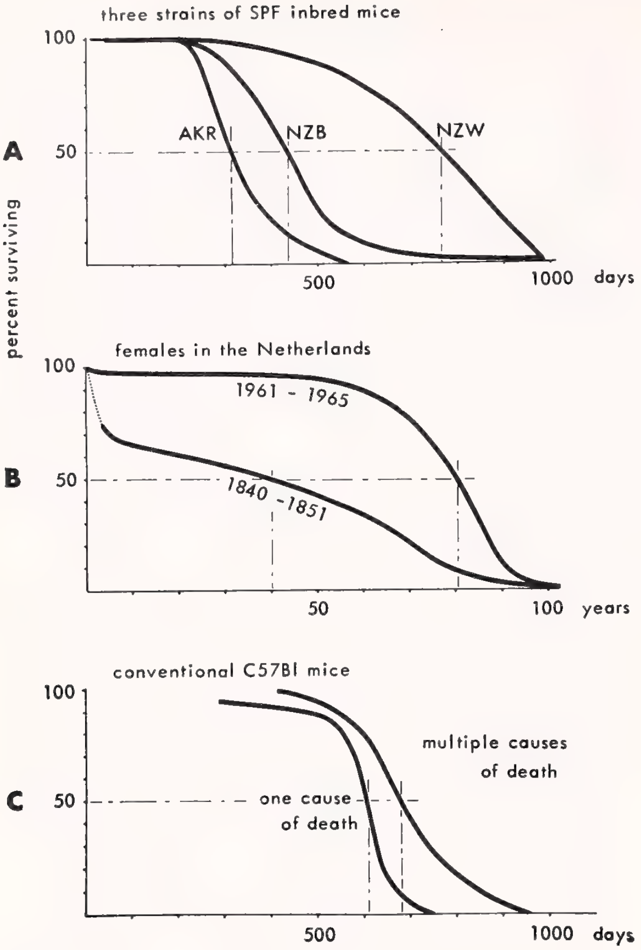
CANCER is a major cause of death for aging humans. The gradual breakdown of immunologic surveillance against neoplasia may be a factor in the increased incidence of tumors in the aged (*1*). There are indications of a decrease in humoral and cellular immune response as animals and humans grow old (*2*). In experimental animals, Teller (*3*) has shown a relationship between aging and spontaneous tumor incidence and between aging and impaired immune response.

Thus we should carefully explore the causal relationships between aging, immunity, and cancer in experimental models. Rodents, with a relatively short lifespan, are traditionally used in the laboratory for cancer research and for aging studies. However, the widely used inbred strains of rodents were developed and selected for cancer research and not for gerontologic study. Two strains of the same species may live to the same age yet show a different slope of mortality curve (text-fig. 1A).

In the examples shown in the text-figure, the curve for the NZB mouse can be explained by the fact that, in this strain, the main cause of death is related to hemolytic autoimmune anemia. In the NZW mouse, as in man, there are multiple causes of death. Furthermore, in some strains a certain type of cancer approaches 100% at a relatively young age [e.g., leukemia in the AKR mouse (text-fig. 1A)]. Intercurrent infections, such as urethral obstruction caused by infectious swelling of the periurethral glands (*6*), may also markedly affect the morbidity and mortality data (text-fig. 1C). This is also true for man (text-fig. 1B). The shift of the survival curve to the right in 1960–65 can be explained by control of acute infectious diseases, better hygienic measures, and improved medical care. The influence of the germ-free state on carcinogenesis and aging has been described (*7*, *8*). The influence of the composition of the microflora on intestinal cancer is under study (*9*).

For studies of aging, we need many animals of good quality. Often we use SPF or even germ-free (GF) animals. The cost of producing and maintaining such animals is an extra financial burden to the already expensive experiments. Furthermore, baseline data on causes of death and incidence of spontaneous tumors must be collected in different strains and species to allow a choice of the appropriate model for further studies (*10*). To ensure that animal models reflect realistic aspects of human aging, the possibility of a spectrum of different malignant tumors should be an integral part of those models.

This raises the question of whether current work on aging in animals can supply information relevant to the problem of aging in man. For rodents, some information is given in most publications: species used, inbred or noninbred strains, number of animals, sex, conventional, SPF, or GF animals, data on housing, diet, and age when killed. Usually, we are not told the maximum age, the 50% survival time, and the natural causes of death. Data on functional aspects of organs or organ systems are also usually lacking. Thus it is



TEXT-FIGURE 1.—Survival curves of: A) 3 strains of specific pathogen-free (SPF) inbred mice (*4*), B) female humans in The Netherlands at 2 different periods (*5*), and C) conventional C57BL mice dying of 1 cause (*6*) and multiple causes (unpublished data).

often hard to find out if an animal killed at, for instance, 24 months, is old, middle aged, or just adult. Therefore, information on the maximum age, number of animals, and the 50% survival time of the strain of animals should be supplied in publications. The calculation of 50% survival time should be based on the number alive at 3 weeks (weaning), since there is often an excess mortality in the first 3 weeks of life. Knowing the 50% survival time, maximum age, and the fraction of animals that reach this age makes it possible to estimate the slope of the mortality curve. With this information, we can draw conclusions about the validity of the term "old" animals. Hopefully, such an analysis will help us evaluate the age and life stage of the animals studied. Of course, optimum information will be provided by mortality curves combined with morbidity data. Standardization of animals would permit interlaboratory comparison of experimental data. Extensive consultation among laboratories is even more valuable when we consider the complexity of the problems raised by long-term investigations. Standardization of animals would be encouraged by provision of information on the stability and composition of the microflora—particularly pathogenic and potentially pathogenic species (*11*).

Finally, in most inbred species, the incidence of certain cancers may approach 100%, whereas other types of cancer may be rare; thus the introduction of noninbred animals may provide less artificial models. *Praomys* (*Mastomys*) *natalensis* might be an example of this. Most strains of this species now maintained are randomly bred. The maximum age reached in our colony is 34 months, and the 50% survival time is 21 months. The species potentially is valuable in cancer research, for the Mastomys is a museum of spontaneous tumors (*12*, *13*). As in man, cancer is a major cause of death in aged Mastomys (unpublished data). Also, Mastomys develops, with age, a wide range of degenerative and other non-neoplastic lesions, such as degenerative joint disease (*14*), renal lesions (*15*, *16*), and thymic lesions associated with symptoms of autoimmune disease (*17*, *18*). No data are yet available on changes in the immunologic system with age, nor have tumor viruses yet been found in the Mastomys. It seems justified, therefore, to explore the suitability of the Mastomys for detailed study of the relationship between aging and cancer—specifically, study of a correlation between age changes in the immunologic system and the occurrence of tumors.

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GUEST EDITORIAL——**by D.B. Clayson**[1]

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Bladder Carcinogenesis in Rats and Mice: Possibility of Artifacts

BLADDER CANCER is one of the more intensively studied experimental neoplasms. Nevertheless, the interpretation of certain aspects of experimental bladder carcinogenesis in terms of the human disease presents many problems. The distinction between benign and malignant lesions of the urothelium may be difficult, as has been discussed [e.g., (*1*)]. The important role of metabolic activation is established (*2*) although never, in the bladder or elsewhere, is the detail fully understood. There are other factors which now appear to have different effects in man and laboratory animals. Discussed here are urolithiasis, certain parasites, and excessive chemically induced inflammation in man, rats, and mice.

BLADDER CALCULI AND BLADDER CARCINOGENESIS

In man, urinary bladder calculi have not been shown to lead to bladder tumors except possibly when they are present in diverticulae (*3*). Dunham et al. (*4*) obtained a weak correlation between bladder stone and bladder cancer but attached little importance to the finding because they were unable to demonstrate whether stone led to tumor or tumor to stone. Furthermore, bladder stones are common in parts of China and in certain other parts of the world, where no excessive frequency of bladder tumors has been reported (*5*).

In rats and mice, there is a correlation between bladder stone and bladder tumor. Strömbeck (*6*) reported papillomatosis of the urinary bladder induced by 2,3′-azotoluene in rats fed a rice diet but not in those fed a conventional European laboratory diet. The papillomas were frequently accompanied by stones, which did not occur when the European diet was fed. Diethylstilbestrol led to bladder tumors in rats, the tumors arising in the presence of stones (*7*). Fitzhugh and Nelson (*8*) found 11 mostly benign bladder tumors in 24 male rats given 2 or 4% diethyleneglycol in their diet. Urinary concretions of calcium oxalate were present. The observation was confirmed by Weil et al. (*9*) who observed 18 bladder papilloma-bearing rats among 89 males receiving treatment, but no bladder tumors in 56 similarly treated females. Calcium oxalate stones were present in male but not female rat bladders. When the calculi were transplanted into the lumen of the bladder of male and female rats not receiving the chemical, tumors nevertheless developed. The probable explanation of these results is that the stone rather than the chemical was responsible for the induction of the tumors. On the other hand, Hueper and Payne (*10*) took a contrary view, concluding from their experiments with polyoxyethylene-8-stearate (a derivative of diethyleneglycol) that diethyleneglycol or some impurity in it was the responsible carcinogen because autopsy of one female mouse that developed a tumor showed no concretion.

Chapman et al. (*11*), by surgical implantation of artificial stones (pellets of paraffin wax) into rat bladders with prepared pouches, showed that the presence of urine is necessary for tumor induction and suggested that the pellet has a cocarcinogenic effect. They stressed that natural calculi growing on the pellet enhanced the tumor yield, but did not indicate whether this was due to the increased size or roughness of the foreign body in the bladder lumen. Specific impurities in the pellet are unlikely to be responsible for the cocarcinogenic effect of pellets, in view of the wide variety of substances capable of inducing bladder tumors after implantation into the lumen of the bladder of mice and rats [(*12*); (*13*), table VII]. The action of the urine as the primary stimulus in Chapman's system (*11*) is not known; it could be a result of small amounts of carcinogens in the urine, as suggested by Hicks (*14*) in another context, or a consequence of bladder function (i.e., the expansion and contraction of the organ during its filling and emptying) (*15*, *16*). Nevertheless, a strong carcinogenic stimulus, a pellet containing 3-methylcholanthrene (12.5%), overrides the need for the presence of urine (*17*).

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Editor's note: Periodically the Journal will publish solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

Technique of Implantation

The technique consists of the surgical implantation of pellets into the lumen of the mouse bladder (*18*, *19*). The pellets consist of a vehicle, such as paraffin wax or cholesterol, alone and with the vehicle incorporating the test substance. The bladder tumor incidences in the control and test groups are usually compared at 40–50 weeks after implantation. Although the results with this test have been consistent [(*13*) p 354], the test has been criticized because substances not demonstrably carcinogenic by other routes of exposure induced tumors.

The limitations of the method have been demonstrated by Jull (*20*) who implanted paraffin pellets into the bladder lumen of mice and then examined the bladder of these mice at intervals. In the first group, killed at 40–50 weeks after implantation, a few low-grade tumors were found. The number of animals with bladder tumors and the tumor malignancy increased with time until 100–110 weeks when ≈50% of the mice had bladder tumors. The implantation technique is thus one of those methods in which the influence of the test chemical is measured against an increasing background incidence of tumors in control animals. With such a method, the incidence of tumors found after the use of a potent carcinogen, such as 3-methylcholanthrene (*21*), is less in doubt than it is after use of a weaker carcinogen such as 2-amino-1-naphthol hydrochloride (*22*). Concomitantly with the foreign body effect, i.e., the ability of the pellet to induce tumors in a functioning bladder, other complications of the technique include differences in the rate of diffusion of the test chemical from the pellet (*23*); the possible decomposition of the test chemical in the pellet; and hydronephrosis, hydroureter, and urinary inflammation and infection in experimental animals.

4-Ethylsulfonylnaphthalene-1-Sulfonamide (ENS)

This drug was synthesized by Imperial Chemical Industries, Ltd. (Great Britain) as a potential diuretic and anticonvulsant (*24*). It induced hyperplasia of the rat urothelium in preclinical trials and was abandoned. My colleagues in Leeds [(*13*), p 284; (*25*)] used it extensively in the study of the properties of the mouse urothelium. The actions of ENS may be divided into 3 groups.

Acute

Intragastric instillation of a single dose in mice (ca. 20 mg/kg) leads to necrosis of the luminal layers of the urothelium and to stimulated DNA synthesis (*26*) with a significant increase in mitosis, and DNA and RNA synthesis (*27–29*). In particular, the urinary pH increases by at least 2.4 pH units (6.1–8.5 U) because the drug inhibits carbonic anhydrase. These effects are presumably due to the sulfonamide group, because 1,4-naphthalenedisulfonamide mimics all the acute effects of ENS (*30*). The acute effect was not counteracted by administration of ammonium chloride in the drinking water. Female mice were quantitatively more susceptible than males.

Subacute

If ENS is fed in the diet at 0.01% (100 ppm) for 4–12 weeks, the alkalinity of the urine is maintained. In (A × IF)F_1 or (C57 × IF)F_1 mice, concretions of calcium ammonium phosphate and oxalate are precipitated and the epithelium becomes hyperplastic. The ultrastructure of the epithelium is considerably modified, and a great disparity in mitotic index is found between different bladders and different parts of the same bladder. If ammonium chloride (1%) is administered in the drinking water with ENS in the diet, the epithelium maintains its normal state and concretions do not form. The changes induced by ENS in the urothelium are mostly reversible if treatment is discontinued at 1 week (*25*), 8 weeks (*31*), or 31 weeks (*32*); reversibility is possibly not complete at 65 weeks (*32*). Female mice are again more susceptible than males.

Chronic

In mice, bladder tumors are found at 33–80 weeks after the administration of ENS (100 ppm in the diet) (*26*). The tumors range in malignancy from papillomas to carcinomas which invade muscle; as many as 45% of females are affected.

The critical question is whether the chemical itself, the bladder stones, or the alkalinity of the urine is responsible for tumor formation. A partial answer has been provided by Flaks et al. (*33*) who demonstrated that, whereas the drug (0.01% in the diet) led to bladder tumors in 27% of mice killed or dying within 1 year, the drug and ammonium chloride (1.0% in drinking water) did not induce bladder tumors. The ammonium chloride corrects the urinary alkalinity arising from the inhibition of carbonic anhydrase and consequently inhibits stone formation. Thus, for the first time, it appears that ENS is active partly through its ability to lead to an alkaline urine and stones in the urinary bladder. Additional mice are being continued on experiment for the remainder of their lives to determine whether ammonium chloride partially or totally inhibits tumor formation by this interesting drug.

Conclusions

The demonstration that artificial and natural calculi in the lumen of rat or mouse bladder may induce tumors [(*12*); (*13*), table VII] is of considerable importance to the interpretation of the genesis of experimental bladder cancer. The wide variety of materials used for bladder implants in such studies includes rough-surfaced glass beads, paraffin wax, hexamethylbenzene, stearamide, calcium oxalate concretions and natural materials such as wood. This militates against, but does not disprove, the idea that the tumors induced by such materials are due to carcinogenic impurities contained within them. If a foreign body in the presence of urine is all that is needed for tumor formation in the rat or mouse urothelium, chemicals provoking urinary calculus formation need careful scrutiny before being accepted as directly carcinogenic.

It follows that in all experiments in which the urinary bladder is studied, it will be mandatory to search for calculi and to record their presence, size, and surface appearance at autopsy. Preferably, urinary pH during the experiment should also be recorded.

If a chemical inducing urolithiasis is tested for carcinogenicity, tumors in tissues other than the urothelium should be used for assessment. If tumors only of the bladder are induced, the substance should be retested under conditions inhibitory to the formation of calculi, or in other systems. In the former case, it may be desirable to design the repeat experiment so that any synergism between the tumorigenic properties of the chemical and the foreign body is demonstrated. To the best of my knowledge, no suitable protocols have been reported which consider that the formation of calculi on implanted pellets increases their (co)carcinogenicity (*11*).

BLADDER PARASITES

Infestation of the human bladder by the digenetic trematode *Schistosomum haematobium* leads to urinary bilharziasis. An increasing body of evidence demonstrates that bladder carcinogenesis is a sequel of infestation (*34–36*). Neoplasia of the urinary tract has been described in nonhuman primates artificially infected with *S. haematobium* (*37*). There is yet no evidence that the parasite introduces a carcinogen into the bladder tissue (*38*).

Trichosomoides crassicauda, the rat bladder threadworm, exists usually in the rat bladder wall and less frequently in the lining of the upper urinary tract. It causes little overt pathologic reaction except slight inflammation. Infection is caused by the ingestion of embryonated ova which hatch in the stomach and after a few hours pass through the stomach wall. They are carried by the bloodstream to all parts of the body, but only those that reach the kidney or urinary tract survive (*39*). This parasite is apparently specific to rats.

The parasitic infection is found among wild or laboratory rats. Chapman (*40*) estimated that a considerable proportion of European rat colonies carry this infection. Infection may be avoided if clean rats are maintained away from infected rats. *T. crassicauda* may be controlled by treatment with methyridine (*41*) or nitrofurantoin (*40*). Nitrofurantoin treatment has not been completely satisfactory (*42*). Although not carcinogenic in itself (*43*), it is so closely related to the potent nitrofuran-based carcinogens that its use is of dubious value in animals intended for carcinogenicity experiments.

Schreiber et al. (*44*) examined the proliferation kinetics of rat bladder epithelium by injecting the radiolabeled specific DNA precursor, tritiated thymidine. In uninfected rat bladders, the labeling index was 0.12±0.01%. When the bladder was infected with *T. crassicauda*, the labeling index of the total epithelium rose to 3%. Irradiation of the uninfected urinary bladder with 2000 rads led to a peak in labeling of 6.5% at 8 days; irradiation with 800 rads led to a lower peak of 3% at 3 days. In both instances, the labeling index fell to near normal levels by the next observation. When the bladders were parasitized, irradiation by 800 rads led to a peak in the labeling index of 18.5% at 8 days, and the index had fallen only to 17% by the end of the experiment (14 days). This experiment clearly indicates that the parasites greatly stimulate the response of urothelial DNA synthesis to irradiation. Unfortunately, similar experiments have not been carried out with bladder carcinogens.

Chapman (*42*) did the only major experiment to clarify the effect of *T. crassicauda* in bladder carcinogenesis. When 2-fluorenylacetamide was fed to rats, there were no bladder tumors in 78 noninfected animals, compared to 5 in 75 infected rats ($0.03 > P > 0.02$). The complexity of the experimental protocol, with 3 strains of rats, both sexes, 2 diets, the presence of tumors in other tissues, and the use of nitrofurantoin in some but not all groups, makes interpretation difficult.

Conclusion

The ability of *S. haematobium* to lead to cancer in the human bladder, whereas *T. crassicauda* has not been demonstrated to do so in rat bladder, is possibly a result of the intensity of the infection or other changes induced by these parasites. The complications induced by *T. crassicauda* in experiments on rat bladder carcinogenesis are unnecessary, since noninfected rats maintained under optimal conditions do not become infected.

SEVERE INFLAMMATION

Oxolamine (3-phenyl-5-β-diethylaminoethyl-1,2,4-oxidiazole) induces bladder tumors by interfering with the bladder epithelium in an apparently different way. Large, single doses of this compound lead to necrotic changes in the bladder epithelium in rats and dogs, but not in mice. Continuous application has been stated to lead to bladder tumors [(*45*), cf.(*46*, *47*)]. This agent has been suggested to liberate alkylamines in the urine as a result of its metabolism (*48*). That various alkylamines (e.g., dimethylamine, diethylamine), when administered by intravenous injection, lead to inflammation of the bladder epithelium suggests that the urinary bladder tumors obtained by Barron (*45*), but not confirmed (*47*), present a further difficulty in the interpretation of the genesis of experimental bladder tumors in rats and mice. This observation apparently has no direct parallel in man.

DISCUSSION

The concept that substances may induce artifacts leading to tumors in animal models is not new. Local sarcomas that arise in response to the repeated subcutaneous injection of hypertonic or surfactant solutions are more likely to be precipitated in response to the physical properties of the solution than chemically induced by the solute (*49–51*). Likewise, the Oppenheimer effect (*52*, *53*), the induction of sarcomas by the subcutaneous implantation of a wide variety of metallic or plastic films in the subcutaneum of rats and mice, is a further example in which the tumor yield depends more on the integrity and total surface area of the film than on its chemical composition.

In the rat and mouse bladder, the incidence of tumors is relatively unaffected by the chemical composition of the artificial or natural calculi, but appears to depend on the normal flow of urine and the size or surface structure of the calculus (*11*). If this interpretation of the data is correct, there should be a similar threshold level of chemical for both urolithiasis and cancer induction.

The tumorigenic effect of urothelial parasitism and chemicals, which induce marked inflammatory effects, cannot yet be assessed. They deserve further study to determine whether they have a significant effect on bladder carcinogenesis.

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ADDENDUM

In the 4 years since this Editorial was published, two other chemicals have been shown to induce stone-associated bladder tumors in rats. According to press reports, 20 or 10% xylitol or 5% terephthalic acid (benzene-1, 4-dicarboxylic acid) added to the diet leads to these conditions.

Of greater importance have been investigations of two-stage systems for rodent bladder cancer. In a 1967 study, my colleagues and I (*1*) demonstrated that a limited nontumorigenic administration of 3-aminodibenzofuran followed after a 2-week interval by the implantation of a paraffin wax pellet led to significantly more bladder tumors than did either treatment alone. Hicks et al. (*2*) claimed originally that one instillation of 2 mg MNU into the bladder lumen did not lead to bladder tumors, but subsequently she and Chowaniec (*3*) and Mohr and his co-workers (*4*) reported a 20–30% incidence under these conditions. Hicks et al. (*5*) claimed that feeding saccharin or cyclamate to MNU-treated rats led to a great increase in bladder tumors. Mohr and co-workers (*4*) were unable to demonstrate any difference in the incidence of rats with tumors of the total urinary tract, nor of the bladder epithelium after instilling MNU and feeding calcium carbonate, sodium saccharin, or calcium cyclamate. Each of these chemicals led to an increase in renal pelvic tumors compared with those found after MNU alone. Therefore, this method has limited use at this time.

Others used a more conventional approach by feeding a limited dose of a potent bladder carcinogen, N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT), and then immediately, or after an interval, adding DL-tryptophan or sodium saccharin (*6*) to the diet. Both of these chemicals, which do not induce cancer when fed to young adult rats, significantly increased the incidence of bladder tumors in FANFT-pretreated rats. This system, therefore, offers researchers the opportunity to investigate the enhancing effect of various agents on urothelial carcinogenesis and to determine the biologic factors concerned in bladder tumor enhancement.

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GUEST EDITORIAL — **Jacqueline Whang-Peng, *M.D.*[1, 2]**

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Banding in Leukemia: Techniques and Implications

The establishment of the normal human karyotype and the universal acceptance of the Denver Nomenclature in 1960 (*1*) marked the beginning of modern human cytogenetics. Since that time, cytogenetic studies as an aid to medical diagnosis have been utilized with great success in the detection of genetic defects. Recently, such studies have been applied to the investigation of presumably acquired diseases, especially neoplastic diseases such as the leukemias. Since increasing evidence exists that at least some malignant cells possess chromosomes with characteristic structural and/or numerical abnormalities, the precise identification of individual chromosomes has become particularly important for the study of human neoplasia.

As of 1960, the 46 human chromosomes could be grouped into eight broad categories (groups A to G and the sex chromosomes) on the basis of their morphology (e.g., size and position of the centromere), which enabled the cytogeneticist to identify with certainty chromosomes #1, 2, 3, and 16. With the introduction of autoradiographic analysis of human chromosomes in the mid-1960's (*2*), it became possible to identify chromosomes #4, 5, 13, 14, 15, and X's in excess of one. This method is based on the labeling of newly replicated DNA with [^{3}H]thymidine. DNA replication does not take place simultaneously along an entire chromosome; instead, different segments of an individual chromosome undergo DNA synthesis at different times during the S-phase of the cell cycle. Therefore, when dividing cells are incubated with [^{3}H]thymidine and then harvested and their chromosomes are examined by autoradiography, each chromosome exhibits a characteristic labeling pattern. This technique makes it possible to distinguish between early- and late-replicating chromosomes and has been used to distinguish one late-labeled (hot) X from other chromosomes of the C group in cells with two or more X chromosomes. Although this technique could also be used to distinguish between pairs of B-, D-, and E-group chromosomes, identification of all the chromosomes in the human karyotype was still not possible.

In 1968, Caspersson et al. (*3*) introduced quinacrine fluorescence staining for the chemical differentiation of metaphase chromosomes. Using this method, one could identify for the first time each pair of chromosomes precisely and characterize details of their structure. This method had the disadvantage, however, of requiring a fluorescent light attachment for the laboratory microscope, which was too expensive for many laboratories. Fortunately, the Giemsa staining technique (*4*) was soon developed and found to be applicable for most laboratory purposes.

MOST FREQUENTLY USED TECHNIQUES FOR IDENTIFICATION OF CHROMOSOMES

The banding method can be applied to morphologic studies not only of fixed chromosomes but also of those in living cells. The two most widely employed techniques for the study of fixed chromosomes are fluorescence and Giemsa staining.

Fluorescence Staining

This technique exploits the differential affinity of various segments along the longitudinal axis of a chromosome for a fluorescent stain; when exposed to UV light, each chromosome shows a characteristic pattern of fluorescent (Q-) bands. The most frequently used stains are quinacrine dihydrochloride (Atebrine), Arancil (Acranil) (*5*), quinacrine mustard (*3*), benzimidazole derivatives (33258 Hoechst) (*6*), and acridine orange (*7*). These stains bind by intercalation between the bases in DNA, and those regions of DNA particularly rich in dA–dT residues are enhanced, thus exhibiting greater fluorescence upon exposure to UV light (*8*).

Giemsa Staining

Chromosomes are stained with Giemsa during or after various chemical and/or physical treatments designed to disrupt nucleoprotein associations. The type of treatment to which the chromosomes are subjected determines the banding pattern produced by Giemsa staining. Presently, four different procedures are in use; they produce characteristic patterns referred to as G-, R-, C-, and T-bands.

ABBREVIATIONS USED: dA-dT = deoxyadenylate-deoxythymidylate; ASG = acetic–saline–Giemsa; CML = chronic myelogenous leukemia; AMML = acute myelomonocytic leukemia; AML = acute myeloblastic leukemia.

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

G-banding.—This procedure is based on the preferential binding of stain to regions of the chromosome containing DNA rich in dA–dT residues. In the most commonly used methods of producing G-bands, ASG (*9*) or the proteolytic enzymes trypsin, pronase, and L-chymotrypsin (*10*) are used, although denaturation with urea (*11*), detergents (*12, 13*), and organic and inorganic salt solutions (*14–16*) also gives satisfactory results. The ASG method, first described by Sumner et al. (*9*), involves fixation of cells in methanol, denaturation of the DNA with acetic acid, and preparation of chromosome spreads by air-drying. The mechanism of this method may be as follows: Nonrepetitive DNA is readily denatured by the acid treatment and has little affinity for Giemsa. A 1-hour incubation in 2×SSC (0.3 M sodium chloride and 0.03 M trisodium citrate) at 60° C allows the annealing of repetitive DNA, which then binds the Giemsa stain to produce characteristic dark bands. In contrast, nonrepetitive DNA anneals imperfectly, if at all, during the incubation, and its location on chromosomes is indicated by the light-staining regions. The technique of proteolytic enzyme (e.g., trypsin) digestion was successfully used by Seabright (*17*) and Wang and Fedoroff (*18*) in 1971. Wang and Fedoroff suggested that the trypsin hydrolyzed the protein component of nucleoproteins after the DNA was denatured by the fixation procedures used. The exposed DNA was then free to react with the Giemsa stain, thereby producing the banding pattern observed.

R-banding.—This technique, described by Dutrillaux and Lejeune (*4*), is based on thermal denaturation of DNA. Air-dried slides are immersed in a phosphate buffer (20 mM, pH 6.5) bath for 10–12 minutes at 87° C and are then stained with diluted Giemsa at room temperature for 10 minutes. The banding pattern of chromosomes is observed by phase contrast microscopy. With the exception of the secondary constriction of chromosome 9, the colored bands correspond exactly to the nonfluorescent regions seen after Q-banding and are opposite in intensity to G-bands.

C (constitutive heterochromatin)-banding.—The procedure involves denaturing of DNA by treatment with HCl, RNase, and NaOH, and then renaturation of DNA in a saline citrate solution at 65° C. The centromeric areas (heterochromatic regions), believed to be composed of the highly repetitive DNA known as constitutive heterochromatin, produce a dark band after Giemsa staining. The facultative heterochromatin regions, such as the repressed X in female cells, do not respond to this stain (*19*).

T (terminal)-banding.—Dutrillaux (*20*) described two methods of T-banding, both of which are similar to the R-banding technique. The first method differs from R-banding by requiring a longer incubation time (up to 30 min). In the second method, the pH of the buffer is reduced from 6.5 to 5.1. After either Giemsa or acridine orange staining, terminal bands can be observed on the chromosomes. The origin of these bands and their exact chemical nature is not known, although it is possible that they are the parts of the R-bands most resistant to the denaturation conditions used. T-banding is especially helpful to precisely localize juxtatelomeric break points.

The relationship between patterns of DNA replication, Q-bands, and G-bands has been examined by Ganner and Evans (*21*). In general, patterns of late replications reflected the patterns of fluorescence and G-bands. The only exception was the heavily labeled (hot) X chromosome in cells from females; this newly synthesized X chromosome could best be identified by autoradiography. Moreover, the patterns obtained after G-banding are almost identical to those produced by Q-banding. The major exception is the Y chromosome; Q-staining reveals brilliant fluorescence on the distal part of the long arm, whereas two distinct bands in this region are produced by the ASG method. Other areas, such as near the centromere of chromosomes #1, 16, and 22, show a dull fluorescence, whereas in G-bands all except #22 show a strong Giemsa-positive region.

NEWER TECHNIQUES OF BANDING IN LIVING CELLS

The banding methods described above all involve the staining of chromosomes after fixation. New banding methods have been developed, however, which allow for the treatment of chromosomes in living cells before harvest, fixation, and Giemsa staining. The substances used, which include actinomycin D (*22, 23*), azure B (*24*), and tetracycline (*25*), all band in the G_2-phase of the cell cycle. The mechanism by which these compounds produce banding patterns on chromosomes is not entirely clear. Actinomycin D is thought to bind specifically to guanine bases in DNA and to compete for a protein-attachment site. Therefore, the light-staining bands observed in chromosomes from actinomycin D-treated cells may result from the displacement of protein from nucleoprotein complexes. The relative content or distribution of adenine–thymine versus guanine–cytosine base pairs along the chromosome may also play a role in the banding patterns produced by actinomycin D. However, actinomycin D, like azure B and tetracycline, possesses planar molecular structures and is able to intercalate between the base pairs of DNA. Such intercalations could disrupt or prevent associations between DNA and protein, thereby allowing the Giemsa or other stain to interact with DNA. The bands produced on chromosomes treated in living cells by actinomycin D, azure B, or tetracycline are identical to G-bands.

At present, most laboratories favor the G-banding technique because it is easy, reproducible, and does not require special equipment. Many laboratories have made minor modifications of the basic technique. In our laboratory, we favor the Giemsa–trypsin banding method, although we use the fluorescence method for some preparations. The methods for Giemsa–trypsin staining and for fluorescence banding used in our laboratory are described below.

Giemsa–Trypsin Staining Method

This method is a modification of the techniques of Seabright (*17*). Store air-dried slides that are 1 week old in a 37° C incubator for 4–5 hours. Immerse the slides patient had hyperplastic marrow and erythroid hyperplasia for a year prior to developing AML.

A missing Y chromosome, easily detected by the Q-banding technique, has been found in leukemias and solid tumors (*65*). To date, at least 28 Ph^1-positive CML patients missing the Y chromosome have been described (*66*), as have fewer cases of Ph^1-negative CML (*67*).

CONCLUSION

The presence of the Ph^1 chromosome in the hematopoietic cells of individuals with apparent hematologic disorders is virtually diagnostic of CML. In patients without any hematologic or clinical symptoms, the presence of the Ph^1 chromosome can be an indicator of the preleukemic state. For example, a small percentage of Ph^1-positive cells was found in the bone marrow of a preleukemic patient 5 years prior to any significant hematologic change and the development of blastic leukemia (*68*). Baccarani et al. (*69*) reported a similar case in which all the metaphases in the bone marrow had a Ph^1 chromosome. Eventually, these cases of Ph^1-positive preleukemia develop a typical leukemic phase.

Published data in cytogenetic studies of acute leukemia (*70–77*) have shown that approximately half the patients exhibit chromosomal abnormalities in their bone marrow. Rowley and Potter (*44*) reviewed the available banding data for acute nonlymphocytic leukemia and noted that the incidence of chromosomally abnormal patients was underestimated by at least 10–20%. They found various nonrandom chromosome changes including an additional #8 chromosome, the loss of chromosome #7, a gain or loss of #21, frequent structural rearrangements of #8 and 21, and the loss of a sex chromosome. Less banding data are available for acute lymphocytic leukemia. In a recent paper on this subject (*78*), I noted that the chromosome group most frequently involved in aneuploidy was the G-group, with the next most common being the B-group. The clinical significance of aneuploid cells in the bone marrow of acute leukemia patients is still unknown. Some investigators feel that the presence or absence of aneuploidy has no apparent bearing on the clinical course or prognosis of disease (*63, 70, 73, 77*), whereas others believe that aneuploidy indicates a poor prognosis (*76*). The presence of aneuploid cells in the bone marrow before and/or after treatment did not necessarily indicate a poor prognosis if adequate treatment was given to eradicate the aneuploid cells (*78*). In fact, aneuploid cells are useful indicators, the disappearance of which suggests successful treatment leading to long-term survival and possible cure. In contrast, the aggressive persistence of aneuploid cells in the bone marrow is a grave prognostic sign. Nowell (*79*) proposed that a positive marrow chromosome study in nonirradiated patients with preleukemic symptoms suggests that a leukemic phase is imminent, whereas a negative study may indicate a protracted nonleukemic course.

Although the new banding techniques appear to be highly useful in cytogenetic studies, relatively little banding data has been accumulated for such leukemia patients. This paucity of banding data is due, at least in part, to a number of technical difficulties encountered in cytogenetic studies of leukemia patients. For example, bone marrow samples from such patients frequently have an abnormally small number of cells undergoing mitosis. In other cases, marrow samples have a predominance of hyperdiploid or near tetraploid cells, yielding too many chromosomes for a good spread. Chromosomes from leukemia patients often have a peculiar stickiness, which also makes it difficult to obtain usable spreads. Some of these difficulties can be obviated with 1-day peripheral blood cultures. These provide a greater number of cells in mitosis, since proliferating leukemic cells are usually abundant in the peripheral blood; moreover, the cells are capable of dividing without mitogenic stimulation. Even when a sufficient number of metaphase spreads is obtained, however, the quality of the banding is frequently poor. This problem is usually due to aged slides or to inadequate banding treatment and can be overcome by application of the banding techniques to relatively fresh (1- to 4-week-old) slides. Preparation of a sufficient number of slides from the same sample is also prudent, so that some steps in the banding procedure can be varied to arrive at an optimal treatment for each individual sample. Finally, improvements in the techniques used to obtain samples will undoubtedly help to resolve many of the problems encountered in cytogenetic studies of leukemia patients.

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while still warm in 0.025% trypsin (Gibco) for a specified time (bone marrow requires from 15 to 30 seconds, peripheral blood from 10 to 20 seconds). Then immerse the slides immediately in cold saline and rinse them well. Stain in 2% Giemsa R66 (G. T. Gurr, #482) in citrate buffer (ph 6.8; G. T. Gurr, #654) for 30–45 minutes. Rinse in tap water and dry.

Q-Staining

This method was described by Caspersson et al. (*26*), and has been modified slightly in our laboratory. To prepare the quinacrine staining solution, dissolve 0.5 mg quinacrine mustard in 10 ml McIlvaine disodium phosphate–citric acid buffer, pH 7.0. Place a few drops of the staining solution on the material to be stained, put a cover slip over it, and stain for 20–30 minutes. Place slides in a Coplin jar, and fill and empty the jar two or three times under running distilled water. Allow the slides to remain in distilled water for 3 minutes, then air-dry. For use, put a few drops of buffer solution or distilled water on the slide, place a clean cover slip over it, and blot the slide with filter paper to remove excess water. Slides are then ready to examine under fluorescent optics.

CLINICAL IMPLICATIONS OF CHROMOSOME BANDING

Much has been learned about chromosome structure and configuration with the use of different banding methods. For example, the Ph^1 chromosome was first described by Nowell and Hungerford (*27*) in patients with CML as a G-group chromosome with a partially deleted long arm. Early in 1973, Rowley (*28*) used fluorescence and Giemsa banding techniques to demonstrate that this marker chromosome actually represents a translocation of the distal portion of the long arm of chromosome #22 to the end of the long arm of chromosome #9. Other workers have described additional translocation sites, including chromosomes #2 (*29*), 6 (*30*), 7 (*31*), 13 (*32*), 17 (*33*), 19 (*34*), and 22 (*35, 36*). From these reports, one may conclude that the deleted portion of the Ph^1 chromosome is usually but not always translocated to chromosome #9.

A new marker chromosome, characterized by a partial deletion of the long arm of chromosome #5 (5q−), was first described by Van den Berghe et al. (*37*) in the bone marrow cells of 3 patients with a distinct type of aregenerative anemia; a total of 6 such cases were studied. By using the Giemsa banding method, Verhest et al. (*38*) were able to determine precisely that the deleted portion was interstitial: del(5) (pter− q12::q31− qter).

We first described the 45 chromosome syndrome in 1964 (*39*) in three patients with refractory anemia, thrombocytopenia, peripheral leukocytosis, and normal-to-low alkaline phosphatase. All cells in their bone marrow contained 45 chromosomes, the missing chromosome belonging to group C. The bone marrow of the patients showed granulocytic hyperplasia with or without megaloblastoid erythropoiesis, and all of them terminated in AMML. A similar case was described by Macdougall et al. in 1974 (*40*); using the Giemsa banding technique, they identified the missing C-group chromosome as #7. Cells from a patient with erythroleukemia (*41*), one progressing from preleukemia to AML (*42*), and another patient with pancytopenia without leukemia (*43*) were also reported to be missing chromosome #7.

The presence of an extra C-group chromosome is one of the most frequently occurring aberrations in leukemia cells. With the new banding methods, the precise identification of this extra chromosome has been possible. Thus an extra #8 chromosome has been found in cases of AML, AMML, and erythroleukemia (*44*), as well as in patients with CML in blastic crisis and in those with polycythemia vera (*45, 46*). In contrast, the extra C-group chromosome has been identified as chromosome #9 in cells from patients with myelosclerosis (*47*), AMML (*48*), and thrombocytosis (*49*); in 2 with polycythemia vera (*50*); and in 1 with massive splenomegaly and active marrow (disease not diagnosed) (*51*).

A long acrocentric chromosome marker was first described by Jacobs et al. in 1963 (*52*) in an African patient with Burkitt's lymphoma, and a similar case was described by Stewart et al. in 1965 (*53*). With the use of Giemsa banding, Manolov and Manolova (*54*) were able to identify this acrocentric marker in 10 of 12 African patients with Burkitt's lymphoma as chromosome #14 with an extra terminal band. However, no such marker has been noted in our studies of American patients with Burkitt's lymphoma. Wurster-Hill et al. (*55*) reported one case each of plasma cell leukemia and multiple myeloma in which two extra terminal bands were present on chromosomes #14. Philip (*56*) reported another case of multiple myeloma with one additional dark band on chromosome #14.

Another specific structural chromosome rearrangement, noted in a wide spectrum of myeloproliferative and lymphoproliferative disorders, is now known to be the isochromosome of the long arm of chromosome #17, designated as i(17q). In a recent review, Engel et al. (*57*) described this chromosome in cells from patients with AML, Hodgkin's disease, and CML as a metacentric chromosome of C-group size. Using ASG banding techniques, Lobb et al. (*58*) conclusively identified this marker as i(17q) in three of their Ph^1-positive CML patients undergoing a blastic crisis. The i(17q) marker has also been found in a case of eosinophilic leukemia (*59*).

Kay et al. (*60*) first reported finding a correlation between an abnormally small F-group chromosome and polycythemia vera. A small F-group chromosome has also been found in cells of patients with various other erythroid disorders, such as idiopathic sideroblastic anemia (*61*), Di Guglielmo syndrome (*62*), AML (*63*), and subacute myeloid leukemia (*61*). We have noted a small F-group chromosome in 5 of our patients with various myeloproliferative and lymphoproliferative diseases and some degree of erythroid dyspoiesis. In 1972, Reeves et al. (*64*) reported that the small F-chromosome in their patients with polycythemia vera was 20q12−. However, of the 4 patients in our series studied by Giemsa banding, only one had a similar marker. This

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